IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Frank Grosveld **Confirmation No. 9062**

Serial No.: **Art Unit No.: 1632**

Filing Date: October 24, 2003 **Examiner: Anoop K. Singh**

For: IMMUNOGLOBULIN 1

Customer No.: 34132

DECLARATION OF DR. FRANK GROSVELD

1. I am the inventor of the above-identified patent application. I have read the Final Rejection dated as mailed July 9, 2007, and would reply to several the issues raised as follows below.

2. The invention describes methods for the in vivo derivation of heavy chain only antibodies in transgenic non-human mammals in response to antigen challenge. The invention requires a modification to the normal mammalian heavy chain locus such that the CH1 domain is not expressed in the heavy chain following the gene activation as a result of antigen challenge in specialised B-cells. In the absence of a CH1 domain the modified heavy chain cannot combine with light chain even if light chain is present (i.e. in a wild type mouse background) and as a result heavy chain only antibody (devoid of CH1) is secreted and circulates in plasma. B-cell specific expression is necessary for a productive response to antigen stimulation leading to VDJ re-arrangement and, ultimately, secretion of antigen specific heavy chain only antibody (devoid of CH1). This would not occur in other cell types (e.g. skin, muscle, heart)

3. To ensure B-cell specific expression of the transgene, human regulatory elements known to induce B-cell specific expression in non-human mammals are present in the natural human IgH sequence described in the application as filed and used by Janssens et al. At the time of the invention, it had already been established for sometime that the insertion of a human heavy chain locus (comprising the CH1

domain) with a human immunoglobulin light chain locus in a mouse background resulted in the in the production of human antibodies in response to antigen. (See Green et al. *Nat Genet* (1994) 7:13-21, "Antigen specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs"; and Gallo et al. *Eur J. Immunol* (2000) 30:534-540, "The human immunoglobulin loci introduced into mice: V (D) and J gene segment usage similar to that of adult humans", copies enclosed). The feature which differentiates this invention, from the prior art, is the absence of a CH1 region. Thus in the cited references the co-expression of light chain was necessary for the productive expression of a normal H2L2 tetrameric antibody from B-cells(light chain constant region binds to the CH1 domain present in the natural immunoglobulin heavy chain). However, as we show, the expression of a human heavy chain gene constructs devoid of CH1 domains allows the functional expression of heavy chain only dimers alone whether the light chain loci are expressed or not. Thus, the elimination of the CH1 domain from any functional heavy chain gene loci and expression in a non-human mammalian background will result in B-cell specific expression of heavy chain only antibody (devoid of CH1) in response to antigen challenge. This is the essence of the invention as exemplified.

4. Based on the knowledge at the time of filing, we expected the regulatory elements present in the constructs used to drive transgene expression in the B-cells of mammals. The additional presence of IgH LCR regulator elements, while not a requirement of the invention, ensures that every insertional event results in a transcriptionally active IgH (devoid of CH1) transgene in every B-cell.

5. In the Final Rejection, the examiner raises questions as to the genetic backgrounds necessary to enable the invention. As we have shown, the genetic background of the mice used in these experiments is irrelevant. There are **preferred** backgrounds in which the endogenous mouse genes are suppressed or eliminated. For example, we have used the μMT mouse. (*See* Kitamura, D., J. Roes, R. Kühn, K. Rajewsky. 1991. Nature 350:423.) In this strain endogenous mouse immunoglobulin gene expression is blocked early in B-cell development so, whilst mouse IgM is detectable, circulating

levels of mouse immunoglobulin are very low. Figure1 shows a FACS analysis of B-cells from the μMT mouse and reveals the presence of low amounts of mouse IgM.

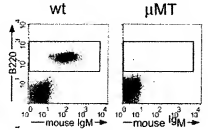

Figure 1 *FACS analysis comparing mouse IgM epression in wild type mice versus μMT mice. The vertical axis shows cell expressing a B cell marker (B220), the horizontal axis cells expressing mouse IgM expression.These panels are copied from Janssens et al 2006, Figure 4A.*

In the absence of significant endogenous mouse IgH gene expression, essentially all plasma immunoglobulin is derived from the introduced heavy chain only transgene. Thus the suppression of endogenous immunoglobulin genes is advantageous, since it facilitates the analysis of heavy chain only gene expression, but is not essential. For example, human immunoglobulin heavy chain only IgM (Figure 2)

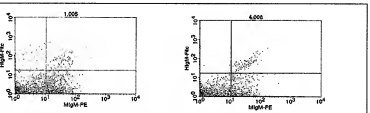

Figure 2 *FACS analysis of heavy chain only IgM expression in a wild type mouse backround.The vertical axis shows cells expressing human IgMfrom the MΔGΔ locus (Janssens et al., 2006), the horizontal axis cells expressing mouse IgM. The panel on the left is a transgenic mouse on a wild type background, the panel on the right a wild type mouse. This figure was not included in Janssens et al 2006*

and human immunoglobulin heavy chain only IgG (Figure 3 copied from Janssens et al 2006) are also expressed from transgenes in normal wild type mouse background.

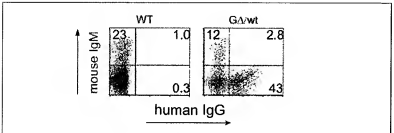

Figure 3 *FACS analysis comparing mouse IgM versus human IgG expression in wild type mice. The vertical axis shows cell expressing mouse IgM, the horizontal axis cells expressing human IgG. The panel on the left is a nontransgenic wildtype mouse, the panel on the right a transgenic mouse on a wild type background. These panels are copied from Janssens et al 2006, Figure 3F.*

To illustrate further the irrelevance of host background to this invention, we enclose details of a human heavy chain only locus where four human V segments replace the two camelid V segments used by Janssens et al. (Fig. 4)

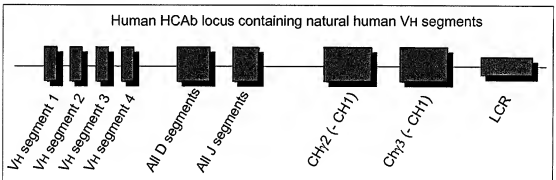

Figure 4 *Scheme of a completely human antibody locus containing natural VH regions. The locus (not to scale) contains the VH3 subclass regions indicated in combination with all of the human D regions, all of the human JH regions, and the human Cγ2 and Cγ3 regions lacking CH1. Lack of the CH1 regions ensures that the locus will result in the production of heavy chain only antibodies.*

This locus is inserted into the chromatin of a wt mouse (FVB strain) where the endogenous murine heavy and light chain genes remain functional. Analysis of serum shows the presence of human heavy chain only antibody (Fig.5 below).

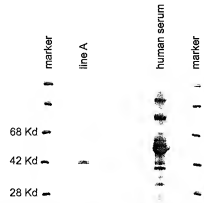

Figure 5 *Western blot showing the expression of human heavy chain only antibody (HAb) in transgenic mice serum containing the 4 natural VH locus (line A) under reducing conditions (i.e. showing single rather than dimer chains although the dimers are visible) . Marker lane shows molecular weight bands, the lane human serum contains normal human IgG. Molecular weights are indicated, the HAb has the predicted size around 40Kd.*

Sequence analysis of human heavy chain only antibody mRNA derived from naïve peripheral B-cells isolated from blood shows that VDJ rearrangement of the transgene occurs as expected in B-cells (Fig.6 below).

EVQLVETGGGLIQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSVIYSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDGLGVAFDIWGQGTMVTVSSER
EVQLVETGGGLIQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSVIYSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARAVAGPYFDVWGQGTLVTVSSER

Figure 6 *Example of two sequences of rearrangements in naïve B cells from a mouse carrying the completely human immunoglobulin locus shown in Figure 4. The largest box shows the CDR3 region, the second largest box the CDR2 region, the box on the right the start of the hinge region going into constant domains and the smallest box inside the CDR2 box a somatic mutation. The sequences show that different rearrangements take place and that somatic mutations are introduced.*

Thus the genetic background used has no bearing on the expression or otherwise of heavy chain only antibody. Any non-human mammal which has a functional IgH locus can therefore be used to express IgH transgenes (devoid of CH1) the object of this invention.

6. The final point I wish to address is the contribution or otherwise of Ledbetter et al. Ledbetter et al. uses a natural endogenous camelid heavy chain only gene loci in the camel in order to generate a functional antigen specific heavy chain only antibody in response to antigen challenge. Thus, V, D, J rearrangement and subsequent affinity maturation occurs in the camel, not in a transgenic non-human mammal. Ledbetter fails to describe, much less generate, a transgenic animal capable of producing heavy

chain only antibody in response to antigen challenge, however. Ledbetter does not enable the derivation of novel heavy chain only antibodies (devoid of CH1) antibodies in the proposed non-human mammalian background as a result of antigen challenge.

7. I hereby declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

13 November 2007

Date

Dr. Frank Grosveld

Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs

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We describe a strategy for producing human monoclonal antibodies in mice by introducing large segments of the human heavy and κ light chain loci contained on yeast artificial chromosomes into the mouse germline. Such mice produce a diverse repertoire of human heavy and light chains, and upon immunization with tetanus toxin have been used to derive antigen-specific, fully human monoclonal antibodies. Breeding such animals with mice engineered by gene targeting to be deficient in mouse immunoglobulin (Ig) production has led to a mouse strain in which high levels of antibodies are produced, mostly comprised of both human heavy and light chains. These strains should provide insight into the adoptive human antibody response and permit the development of fully human monoclonal antibodies with therapeutic potential.

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Fully human antibodies, with lower immunogenicity and more desirable pharmacological properties than engineered mouse antibodies, may fulfill the enormous potential for monoclonal antibodies (mAbs) in treating human disease. As the use of human B cells as a source of rearranged human antibody genes may limit the generation of therapeutically useful specificities, particularly when the target antigen is of human origin, attention has focused on the use of transgenic mice bearing unrearranged human immunoglobulin (Ig) genes to exploit the adaptive immune response of the mouse[1–4]. So far, however, the ability to generate antigen-specific human antibodies in mice has proved elusive. Mice bearing minigene constructs rearrange and express human Ig genes, but the highly skewed or aberrant, fetal-like human Ig repertoires produced in such mice, and the low expression of human Ig relative to endogenous mouse Ig[2–4], has precluded the demonstration of antigen-specific human antibodies. Hence the need for large germline segments of human Ig genes with larger variable gene repertoire and critical regulatory elements to achieve normal levels of expression and diversity is suggested.

Here we describe a novel strategy which permits the generation of mouse hybridomas making antigen-specific human mAbs and the creation of a mouse strain in which the majority of Ig produced are fully human. Using technology we have recently developed[5], yeast artificial chromosomes (YACs) carrying large segments of the human heavy and κ chain loci have been introduced into the mouse germline via fusion of yeast spheroplasts with mouse embryonic stem (ES) cells. These mice produce a broad adult-like repertoire of human Ig and are capable of giving rise to antigen-specific human mAbs upon immunization. Breeding of such mice with those whose heavy and κ genes have been inactivated by gene targeting has led to the creation of a strain which primarily produces fully human antibodies. Such mice may be exploited to elucidate the nature of the human humoral immune response upon infection or immunization, and to develop fully human therapeutic mAbs.

Human Ig YACs in ES cells and mice

YACs containing sequences from the human heavy and kappa chain loci (Fig. 1) were shown to be in intact, germline configuration (M.J.M. *et al.*, manuscript in preparation). The cloned heavy chain YAC (220 kb insert) contains the mu (μ) and delta (δ) constant (C) regions, all six functional joining (J) regions, the major diversity (D) cluster, the intronic enhancer and five most proximal variable (V) genes from four V_H families: V_{H3}, V_{H2}, V_{H3}, $V_{H7.4}$ and V_{H5} (Fig. 1)[6,8]. The cloned κ YAC (170 kb insert) contains the κ deleting element (Kde), the intronic and 3′ enhancers, the $C_κ$ region, all five functional J regions and the three most proximal $V_κ$ regions in the B cluster (B1, B2, B3) (Fig. 1)[10]. A human HPRT selectable marker[11] was targeted into the right vector arm of each YAC (Fig. 1; M.J.M. *et al.*, manuscript in preparation).

HPRT-targeted human heavy (yH1) and κ (yK1) chain YACs were introduced into the HPRT-deficient ES cell line E14.TG3B1 (H.T. *et al.*, manuscript in preparation) by yeast spheroplast-ES cell fusion[5]. Seven and 11 $HPRT^+$ ES clones obtained by fusion of yH1- or yK1-containing yeast, respectively, were analysed by Southern blotting for the integrity of the YACs. Five yH1-containing clones (2B, 2C, 3A, 125A, 125E) and 10 yK1-containing clones contained all *Hin*dIII fragments detected by probes

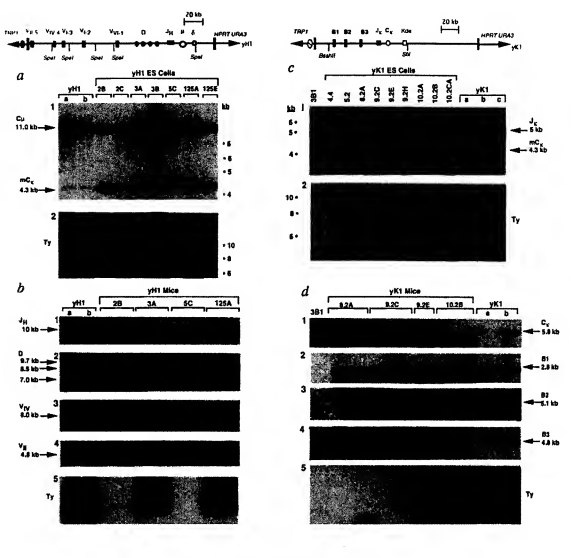

Fig. 1 Characterization of human heavy and κ light chain YACs integrated in ES cells and transgenic mice. Schematic representations of the human heavy (yH1) and kappa (yK1) YACs, retrofitted with a HPRT minigene, are shown above *a* and *c*, respectively. The locations of specific Ig regions are indicated along with YAC vector elements: ▶, telomere; ○, centromere; ▮, *Eco*RI cloning site and yeast selectable markers, TRP1 and URA3. (YAC arms are not shown to scale.) *a*, *b*, Southern blot analysis of *Hin*dIII-digested DNA (10 μg) from: *a*, yH1-containing ES clones: 2B, 2C, 3A, 3B, 5C, 125A and 125E, probed with human Cμ and mouse Cκ (*a1*) and yeast Ty sequences (*a2*); *Hin*dIII-digested yH1, embedded in agarose, was used in *a*, resulting in slightly slower-migrating fragments than the corresponding fragments from yH1 in ES cells prepared in solution. *b*, mice (2 individual offspring from each strain) generated from clones: 2B, 3A, 5C, 125A, and yH1-containing haploid yeast DNA (a= 40 ng and b=20 ng corresponding to 2 and 1 YAC DNA copies, respectively). Probes: J_H (*b1*), D (*b2*), V_H (*b3*, the band detected above the 8 kb V_H fragment in the 2B lane represents a partial digest), V_γ (*b4*) and yeast Ty sequences (*b5*). *c*, *d*, Southern blot analysis of *Hin*dIII-digested DNA (10 μg) from: *c*, unmodified E14.TG3B1 (3B1) and yK1-containing ES clones: 4.4, 5.2, 8.2A, 9.2C, 9.2E, 9.2H, 10.2A, 10.2B and 10.2CA and *d*, mice (2 individual offspring, except 9.2E) generated from clones 8.2A, 9.2C, 9.2E and 10.2B, yK1-containing haploid yeast DNA (a=10 ng, b=20 ng and c=40 ng (when shown), representing 0.5, 1 and 2 YAC DNA copies, respectively). Probes: J_κ and mouse C_κ (*c1*, *d1*), B1 (*d2*), B2 (*d3*), B3 (*d4*) and yeast Ty sequences (*c2*, *d5*). Fragment sizes (in kb) are indicated. *e*, Identification by PCR analysis of HuAb mice (1, 3 and 10) in a mouse litter (1–12) derived from the mating of yH1- and yK1-bearing mice. Specific human heavy chain (D, 230 bp), κ chain (J_κ, 860 bp) and mouse γ interferon receptor (INF-γR, 550 bp)-PCR products are indicated in control 129×C57Bl/6 mice (C), heavy (yH1)- or κ (yK1)-containing mice, or yH1;yK1 progeny.

Table 1 Structural integrity of human heavy and κ light chain YACs in ES clones and their expression in mice

a

yH1 ES Cell Clone	Cδ 7.8 kb	Cμ 11 kb	JH 10 kb	D 9.7, 8.5, 7 kb	VVH ~28 kb	Vl >12, 1.7 kb	VlV 8 kb	VlI 4.8 kb	Yeast Genomic Sequences	YAC copy number
2B	+	+	+	+	+	+	+	+	-	1
2C	+	+	+	+	+	+	+	+	+	2
125A	+	+	+	+	+	+	+	+	+	1
125E	+	+	+	+	+	+	+	+	-	1
3A	+	+	+	+	+	+	+	+	+	1
3B	+	+	+	9.7 kb 8.5 kb	+	+	+	+	+*	~8
5C	+	+	+	+	+	+	+	-	-	1

b

yK1 ES Cell Clone	Kde 2.5 kb	Cκ 5.8 kb	Jκ 5 kb	B3 4.8 kb	B2 5.1 kb	B1 2.8 kb	Yeast Genomic Sequences	YAC copy number
4.4	+	+	5.5 kb	4.7 kb	4.9 kb	+	+	1
5.2	+	+	+	+	+	+	-	1
8.2A	+	+	+	+	+	+	-	1
9.2E	+	+	+	+	+	+	+	2
9.2C	+	+	+	+	+	+	-	1
10.2B	+	+	+	+	+	+	+	1
9.2H	+	+	+	+	+	+	-	~3
9.2F	+	+	+	+	ND	+	-	1
10.2C	+	+	+	+	ND	+	-	1
10.2A	+	+	+	+	+	+	+	2
10.2CA	+	+	+	+	+	+	+	2

c Human Heavy Chain

yH1 Mouse Clone	yH1 Copy Number	hμ Expression (μg/ml)
*3B	~8	3.6
125A	1	0.9
5C	1	0.8
2B	1	0.7
3A	1	0.4
2C	2	0.4
*125E	1	0.2
Control	0	0.0

d Human Kappa Chain

yK1 Mouse Clone	yK1 Copy Number	hκ Expression (μg/ml)
5.2	1	30.0
10.2B	1	27.5
9.2C	1	17.7
*9.2H	~3	15.5
*9.2F	1	11.0
9.2E	2	10.5
*10.2C	1	8.1
8.2A	1	8.0
4.4	1	0.0
Control	0	0.0

a, *b*, HPRT⁻-ES clones, generated by fusion of ES cells with yH1- (*a*) or yK1- (*b*) containing yeast spheroplasts, were analysed for the presence of human heavy or κ chain-specific sequences, and yeast genomic sequences (see Methodology). The presence of the expected fragment sizes for the specific probe (as shown), and of varied levels of yeast genomic sequences are indicated as '+'. Altered-sized fragments are indicated. In the case of yH1- ES clone 3B, the 7 kb doublet from the D region was deleted. ND-not determined. *c*, *d*, Detection of human heavy and κ chains in mouse sera. Serum samples derived from yH1- (*c*) or yK1- (*d*) containing transgenic or chimaeric (*) mice, from the indicated ES clones, or non-transgenic littermate mice (control), were analysed by ELISA for hμ or hκ. Shown are representative serum titrations for individual mice.

spanning the entire respective inserts (Fig. 1, Table 1). Deletions within the D or V_H regions of yH1 were detected in clones 3B and 5C, respectively, and altered Jκ, B3 and B2 fragments of yK1 were detected in clone 4.4. All clones retained the HPRT-containing right vector arm. All yK1-containing clones and four out of seven yH1-containing clones (2C, 3A, 3B, 125A) retained an intact left arm (data not shown). All clones contained a single YAC integration except 2C and 3B (yH1) and 9.2E, 9.2H, 10.2A and 10.2CA (yK1). Hybridization with yeast repetitive probes (Ty, Y', δ, rRNA) demonstrated the presence of varying amounts of yeast genomic sequences in 4 yH1 ES clones (2C, 125A, 3A, 3B) and 5 yK1 clones (4.4, 9.2E, 10.2B, 10.2A, 10.2CA) and their absence in the remaining ES clones (Fig. 1, Table 1).

The integrity of introduced YACs was further assessed by pulsed-field gel electrophoresis following digestion with *Not*I (a site absent in yH1) or *Sfi*I (a site occurring once in yK1, generating a 105 kb fragment containing the Kde-B1 region). A single, unique fragment hybridizing to multiple yH1 or yK1 specific probes was demonstrated in 5 yH1 (2B, 3A, 5C, 125A and 125E) and in 6 yK1 (5.2, 8.2A, 9.2C, 9.2F, 10.2B, 10.2C) ES clones (data not shown; M.J.M. *et al.*, manuscript in preparation). Together, these results strongly suggest that these clones contain a single, structurally intact YAC.

YAC-containing chimaeric mice were generated from 7 yH1 and 9 yK1 ES clones. Approximately 50% of their agouti offspring contained in their germline the respective YAC and yeast sequences (when present) in unaltered form (Fig. 1).

Human Ig production in mice

The expression of the human heavy mu (hμ) and kappa (hκ) chains on B cells and in serum of yH1- and yK1-containing mice was investigated by flow cytometry and

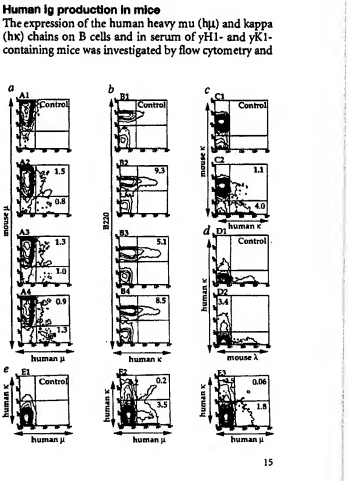

Fig. 2 Surface expression of human μ and κ chains on yH1- and yK1- containing mouse B cells. Blood or spleen lymphocytes derived from yH1-(*a*), yK1-(*b*,*c*,*d*) and yH1;yK1- (HuAb) (*e*) containing mouse strains or control mice (129xC57Bl/6:A1, B1, C1, D1, E1) were analysed by 2- or 3-colour flow cytometry for surface expression of human μ or κ chains, using antibodies to the B cell-specific marker B220 in combination with anti-human μ or κ, and anti-mouse μ, κ or λ, respectively. The net percentage of positively-stained cells (obtained by subtracting the background staining of each control) is shown in each quadrant. *a*, Blood samples from control (A1), or yH1- mice generated from clones 2B (A2), 5C (A3) and 125A (A4), were gated on B220⁺ cells and assayed for mIgM⁺ and mIgM⁺, and hμ. *b*, Spleen lymphocytes from control (B1) or yK1-containing mice: 9.2C (B2), 8.2A (B3), and 10.2B (B4) were assayed for B220 and hκ. *c*, *d*, Spleen lymphocytes from control (C1, D1) or yK1-containing mice 8.2A (C2, D2), gated on B220⁺ cells and assayed for expression of hκ and mκ (*c*) or gated on B220⁺ cells and assayed for expression of hκ and mλ (*d*). The percentage of mλ⁺ B cells was similar (approximately 6%) in D1 and D2. *e*, Blood samples derived from control (E1) or HuAb strains (E2: 8.2A;125A, E3: 8.2A;5C) were analysed by 3-colour flow cytometry for surface expression of human μ and κ on B220⁺ cells. The net percentage of positively-stained cells is shown in each quadrant. The FACS profiles shown are representative of five experiments performed on these strains. Similar analysis indicated the presence of hμ⁺/hκ⁺ populations in other HuAb strains: 8.2A;2B (0.27%), 9.2C; 125A (0.13%) and 9.2C;2B (0.11%).

Human Ig production in mice

The expression of the human heavy mu (hμ) and kappa (hκ) chains on B cells and in serum of yH1- and yK1-containing mice was investigated by flow cytometry and

Table 2 Repertoire analysis of human heavy chain transcripts expressed in transgenic mice

a

Clone	Frame	V_H	D_H	J_H	N	N	J_H	J_H
μ40	in	V_H4	GCGAGAGA	YCG	H1	GGGTATAG	YCGGACCC	J4	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ43	out	V_H4	GCGAGAGA	GGA	H1	GGTATACGACgtgGC	CGGTACA	J3	CTTCGATATCTGGGCCAAGGGACAATGGTCACCGTCTCTTCA
μ46	in	V_H4	GCAA		23/9	TTACTATGATAGTATAGTGGTTATTAC	TGACCATTACT	J4	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ50	in	V_H1	GCGAGA	GGG	2P4	CGATTTTTGGAGTGGTTA	AGCAGGTCC	J6	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ55	in	V_H1	GCGAGAGA	GAGGC	H1	GTATACTCGGAACTAC	GGAG	J6	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ56	in	V_H4	GCA		H1	GGTATACTCGGAACT		J6	AACTACGTCCACCGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ65	in*	V_H1	GCG	GTCC	2P3	GTATTACGATATTTTGACTGGTTA	CCCCTGAT	J3	GCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCA
μ73	in	V_H4	GCG	C	E1	GTCGATATATGTAGCTGCGAT		J3	GCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCA
μ80	out	V_H4	GCAGAGCA		H1	ACTCGAACTAC	CGGG	J4	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCCTCA
μ85	in	V_H1	GCGAG	GGGCGG	H1	TACACGtcgGCTGCTAC	CTCA	J4	TTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCCTCA
μ87	in	V_H1	GCGAGAGAG		H4	TATACGACGCTCGTC		J4	CTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCCTCA
μ89	in	V_H1	GCGAGA	AGGCCG	H1	AGCACGACGT	TCCC	J4	CTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCCTCA
μ90	in	V_H1	GCGAGAG	GAGGGGCCCATT	LB2	GTGGTGGTtgaCTGGCT	CTACGTAC	J4	TACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ97	in	V_H4	GCGAGA		LB2	GTGGgaCTT	ACCCT	J4	CTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ98	out	V_H4	GCAAGAGA	TGAGGGGA	A4	TGACTACAGTAAC	CC	J4	GGGCCAGGGAACCCTGGTCACCGTCTCCTCA
μ100	in	V_H4	GCAAGAGA	GGGACAGTGGC	21/9	TGGTTATTAC		J6	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ102	in	V_H4	GCAAGAGA		H1	TACTCGAAC	ACGT	J6	CTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
μ104	in	V_H4	GCA		A1	GACTACAGTAACT		J4	TTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA

b

Clone	V_H3	CDR3	J_H4
μ40	AR	DRGIVGARYDY	WGQGTLVTVSS
μ46	AR	TRITVVITSDYYFDY	WGQGTLVTVSS
μ50	AR	GRTLEMLESYYYYGMDV	WGQGTTVTVSS
μ55	AR	ERSTTGYYEYYYYGMDV	WGQGTTVTVSS
μ56	AG	IYGTMWFDP	WGQGTLVTVSS
μ73	AR	GYSGYGAVDI	WGQGTMVTVSS
μ85	AR	GGSSGMYLIDY	WGQGTLVTVSS
μ87	AR	ETSSSIFDY	WGQGTLVTGSS
μ89	AR	RASSPSFDYV	WGQGTLVASSS
μ90	AR	GGAGCGGCSYYIYFDY	WGQGTLVTVSS
μ97	AR	VGATLFDY	WGQGTLVTVSS
μ100	AR	RGAVAGYYYYYYGMDV	WGQGTTVTVSS
μ102	AR	DMWTDYYYWYV	WGQGTLVTVSS
μ104	AR	YDFFDY	WGQGTLVTVSS

c

	V_H2	V_H4	V_H1	V_H3	Total	Human PBL(ref.13)
2_3	0	0	1	0	1 (1%)	1%
2_2	0	0	1	0	1 (1%)	0%
2_B	0	9	7	3	18 (18%)	8.1%
J_4	0	8	16	21	45 (54%)	52.1%
2_5	0	0	3	1	4 (5%)	15.2%
J_6	0	3	3	7	13 (13%)	23.3%
Total	0 (0%)	20 (19%)	33 (32%)	32 (29%)	85 (100%)	100%

Human μ-specific mRNAs were amplified by PCR, cloned and analysed by sequencing or by hybridization to V_H- and J_H-region specific probes. *a*, Nucleotide sequences of 18 unique human heavy chain clones are divided into V_H, D, J_H and N segments, as identified by homology with published germline sequences[6,12,28-31]. Whether each V–D–J junction is in or out of open reading frame is indicated. Each D segment assignment is based on at least 8 bases of homology. Differences from the published sequences are in lower case. N-segment nucleotides were determined by their lack of sequence homology to V_H, D or J_H sequences. Clones chosen for sequencing had previously been shown to possess a V_H segment by colony hybridization (see part *c*). *b*, Predicted amino acid sequences of the 14 in-frame V–D–J junctions are divided into Framework Region 3 (FR3), CDR3 and FR4[14]. *c*, Results of colony hybridizations showing V_H and J_H gene utilization, as compared to that detected in human peripheral B cells[13]. Results are given only for colonies hybridizing to V_H, J_H and Cμ probes.

ELISA. Hμ was expressed on the surface of 1.5–2.5% of the B220$^+$ cells in blood (Fig. 2*a*) or spleen (not shown) in all 5 γH1 strains analysed (2B, 2C, 3A, 5C, 125A). Approximately half of the hμ$^+$ population had no detectable surface mouse mu (mμ) (Fig. 2*a*), indicating that hμ production can substantially exclude the expression of mμ. Hμ was detected in sera derived from all γH1 strains at 0.2–3.8 μg ml^{-1} (Table 1*c*). In all 3 γK1 strains examined (8.2A, 9.2C, 10.2B), 5–9% of the B220$^+$ splenocytes expressed surface hκ (Fig. 2*b*). In >80% of this population, hκ excluded the expression of mouse lambda (mλ) or kappa (mκ) (Fig. 2*c*,*d*). All γK1 mice derived from structurally intact YACs expressed hκ in serum at 8–30 μg ml^{-1} (Table 1*d*).

Human Ig-producing (HuAb) mice containing one copy each of γH1 and γK1 were generated (Fig. 1*e*). Three-colour flow cytometry of peripheral blood lymphocytes, derived from five HuAb strains (8.2A;125A, 8.2A;5C, 8.2A;2B, 9.2C;125A, 9.2C;2B) revealed B220$^+$ populations containing 0.8–3.9% hμ$^+$ or 1.4–4.8% hκ$^+$ cells (Fig. 2*e* and data not shown). Significantly, all five HuAb strains contained a population of B220$^+$ cells (0.06–0.27%) which simultaneously expressed both hμ and hκ (Fig. 2*e* and legend). The production of serum antibodies in HuAb mice containing both hμ and hκ (hμ/hκ) was demonstrated by an ELISA in which anti-hμ antibody was used to capture and anti-hκ antibody used to detect the product. Hμ/hκ antibodies were detected in HuAb strains 8.2A;2B and 8.2A;5C at 0.7 and 0.3 μg ml^{-1}, respectively (data not shown). Thus, in HuAb mice, human heavy and kappa genes on YACs were productively rearranged and expressed, leading to the presence of a significant population of B cells expressing both surface hμ and hκ and secreting antibodies containing both human heavy and light chains in the mouse serum.

Diverse adult-like human Ig repertoire in mice

To determine the diversity of the hμ and hκ repertoire in YAC-containing mice, hμ and hκ cDNAs were cloned from mouse spleen RNA. Hybridization analysis of hμ and hκ cDNA clones revealed broad usage of the V and J genes contained in the YACs (Tables 2*c*, 3*c*). All six J_H and five J_K segments were represented with a frequency comparable to that detected in adult human B cells[12,13]. Three of the four V_H families in γH1 were represented, with V_{H1} used about half as often as V_{H3} and V_{H2}. No V_{H4} transcripts (Table 2) or V_H–J_H rearrangement products (data not shown) were detected, suggesting that the proximity of V_H to the YAC vector cloning site may account for its inability to rearrange. All three V_K genes were represented, with B3 more frequently used than B2 or B1 (Table 3).

To analyse further the human Ig repertoire expressed in these mice, individual cDNA clones were sequenced. The 18 hμ cDNAs analysed displayed a V_H and J_H usage similar to that detected by hybridization (Table 2). Ten different D regions were represented, all with significant homology to known germline D genes. Some D sequences with less than 85% identity (for example, N1 in clones μ43 and μ85, and LR2 in clones μ90 and μ97) may represent novel D segments. For example, the C to T and A to G changes observed at the same position in the D segments of μ43 and μ85 (and ref. 12), suggest the existence of a new DN family member. Multiple reading frames were used in some of the D regions (μ90/μ97, μ55/μ102, μ46/μ100), suggesting a D usage more human-like than mouse[12,14]. Non-germline nucleotides (N addition) were observed in 15 (83%) of the hμ cDNAs, with a majority having N additions at both the VD and DJ junctions. The length of N addition varied between 1–12 bp (average 6.1). The majority of the in-frame clones contained a complementarity determining region 3 (CDR3) of 10–18 amino acids (average 12). The $V_κ$ and $J_κ$ usage observed for 21 sequenced hκ cDNAs also agreed with hybridization analysis. In seven of the clones, N additions of 1–4 bp were found at the V–J junction. CDR3 sequences for in-frame κ transcripts were 9–10 amino acids in length. The $J_κ$ usage and CDR3 length observed are consistent with previous results for human B cells[13].

The pattern of V_H, D and J_H usage observed in human Ig YAC-containing mice is reminiscent of adult human B cells[12,13], in contrast to human Ig minigene-bearing mice[2–4]. There is an absence of position-biased V_H and D usage, in particular, for the V_H and DQ52 segments which are characteristic of human fetal development[15,16], with the latter dominating the in-frame repertoire observed in minigene-bearing mice[3,4]. Furthermore, the average length of N addition (6.1 bp), and thus the CDR3 region, closely approximates that seen in adult human B cells (7.7 bp)[12], while in minigene-bearing mice the average length (2.9 bp)[3,4] resembles that seen in adult mouse B cells (3.0 bp)[17]. These results suggest that the human Ig YACs contain sequences required to direct human-like repertoires in mice. Therefore, introduction of YACs

Table 3 Repertoire analysis of human κ transcripts expressed in transgenic mice

a

Clone	Frame	V	N	J	
K1	in	B3	AGTACTCCTC	J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K2	out	B3	AGTACTCCT	TTCT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K3	in	B3	AGTACTCC	J1	GTGGACGTTCGGCCAAGGGACCATGGTGGAAATCAAACGA
K5	out	B3	TTTTCCTC	J3	ACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
K7	out	B3	AGTACTCCCT	J1	GACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K8	in	B3	AGTACTCCTC	J2	GCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K9	in	B3	AGTACTT	T	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K10	in	B3	AGTACTCC	J1	GTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K13	out	B3	AGTACTCCTC	G	J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K14	in	B3	AGTACTCC	CAT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K15	out	B3	AGTACTCC	AT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K16	out	B2	TTCCCTC	J3	ATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
K18	in	B3	AGTACTCC	J4	GCTCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K20	in	B2	TTCCCT	J1	TGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K22	out	B2	TTCCCTC	J4	ACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K25	in	B3	AGTACTCC	J3	ATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
K27	in	B3	AGTACTCC	J4	GCTCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K28	out	B3	AGTACT	GTC	J3	TCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
K29	in*	B3	AGTACTCC	CAT	J2	GTGCAGTTTTGGCCAGGGGACCTTGCTGGAGATCAAACGA
K30	in	B1	TTTCCTC	J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K31	out	B3	AGTACTCCTCC	J4	ACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K32	in	B3	AGTACTCC	J3	ATTCACTTTCGGCCCTGGGATCAAGTGGATATCAAACGA
K33	out	B3	AGTACTCCTCC	J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K34	in	B3	AGTACTCCTCC	J1	GACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA

b

Clone	FR3	CDR3	FR4
K1	YYC	QQYYSTPRT	FGQGTKVEIKR
K3	YYC	QQYYSTPWT	FGQGTKVEIKR
K8	YYC	QQYYSTPRS	FGQGTKLEIKR
K9	YYC	QQYYSTLCS	FGQGTKLEIKR
K10	YYC	QQYYSTPWT	FGQGTKVEIKR
K14	YYC	QQYYSTPMCS	FGQGTKLEIKR
K18	YYC	QQYYSTPLT	FGQGTKVEIKR
K20	YYF	CLQHDNFPWT	FGQGTKVEIKR
K25	YYC	QQYYSTPPT	FGPGTKVDIKR
K27	YYC	QQYYSTPLT	FGQGTKVEIKR
K30	YYC	LQSKNFPRT	FGQGTKVEIKR
K32	YYC	QQYYSTPPT	FGPGIKVDIKR
K34	YYC	QQYYSTPPT	FGQGTKVEIKR

c

	B1	B2	B3	Total
J_1	4	13	108	125 (47%)
J_2	10	5	53	68 (26%)
J_3	0	2	29	31 (12%)
J_4	1	9	25	35 (13%)
J_5	0	0	4	4 (1%)
Total	15 (6%)	29 (11%)	219(83%)	263 (100%)

mRNAs containing $hC_κ$ were amplified by PCR, cloned and analysed by sequencing or by colony hybridization to $V_κ$- and $J_κ$-region specific probes. *a*, Nucleotide sequences of V–J junctions of 21 independent human κ clones are shown, divided into $V_κ$, $J_κ$ and N segments and identified based on homology to published germline B1, B2 and B3, and $J_κ$ sequences[26–28]. Also indicated is whether each V–J junction is in or out of an open reading frame. N-segment nucleotides were determined by their lack of sequence homology to neither $V_κ$ nor $J_κ$ sequences. Differences from the published sequences are in lower case. The sequences shown are those which contained a V–J joining. The one sequenced B1-containing clone lacked a translational initiation site, as described[25]. *b*, Predicted amino acid sequences of in-frame V–J junctions are divided into FR3, CDR3 and FR4[14]. *c*, Results of colony hybridizations showing $V_κ$ and $J_κ$ gene utilization. Results are given only for colonies hybridizing to $V_κ$ and $J_κ$ probes.

with larger numbers of variable genes should ultimately recapitulate the diversity seen in humans.

Ag-specific fully human mAbs from mice

To determine whether HuAb mice can mount a specific human antibody response, mice were immunized with tetanus toxin C fragment (tet C). After immunization, tet C-specific hμ and hκ were readily detected in serum (Fig. 3*a*). The human origin of the tet C-specific antibodies was confirmed by using an ELISA in which tet C was used to capture and anti-hμ or anti-hκ used to detect the bound species. Thus, upon immunization, the HuAb mice are capable of producing antigen-specific human antibodies.

To determine whether antibodies containing both human heavy and light chains were produced, splenocytes derived from tet C-immunized HuAb mice (8.2A;5C) were fused with P3X63-Ag8.653 myeloma cells, and the resulting hybridomas screened for the production of tet C-specific fully human antibodies. Analysis of 678 hybridoma culture supernatants revealed 92 hκ$^+$ clones and 16 hμ$^+$ clones. Three clones were found to produce fully human mAbs specific for tet C. To confirm that all of the desired properties reside within the same antibody molecule, ELISAs were used in which either tet C was used to capture and anti-hκ used to detect the bound species (Fig. 3*b*), or anti-hμ used to capture and anti-hκ used to detect the bound species (Fig. 3*c*). All three clones were positive in both assays, indicating that HuAb mice, although containing only a small fraction of B cells co-expressing hμ and hκ, can mount an antigen-specific response leading to the generation of fully human mAbs. The nature of the human repertoire associated with the tet C-specific response and the extent to which somatic mutation plays a role in the maturation of tet C-specific human antibodies is currently under investigation.

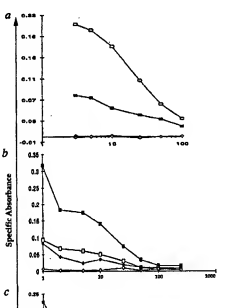

Fig. 3 Production of tet C-specific human polyclonal (*a*) or monoclonal (*b, c*) antibodies by HuAb mice. *a*, Sera obtained from HuAb strain 8.2A;125A, non-immune (◇, ◆) or hyperimmune to tet C (□, ■) were analysed by ELISA for tet C-specific hμ (□) and hκ (■) antibodies. Similar results were obtained from HuAB strains 8.2A;2B, 8.2A;5C and 9.2C;2B. *b,c*, Supernatants derived from hybridomas (42C7-■,45B5-□, 45D9-◆, control 37G4-◇), generated by fusion of hyperimmune HuAb strain 8.2A;125A splenocytes with mouse myeloma cells, were analysed for the presence of fully (hμ/hκ) human monoclonal antibodies (*b*) and for specificity of the human antibodies to tet C (*c*), as detected in ELISA using tet C to capture and anti-hκ to detect. The hybridoma 37G4 (◇), secreting a tet C-specific monoclonal antibody containing only human heavy chain was used as a control in *b, c*.

Human YACs restore B cell and Ig production

While HuAb mice can mount an antigen-specific human antibody response, the preferential expression of mouse Ig genes suggested the potential value of their inactivation to increase the production of fully human antibodies in mice. Therefore, yH1- and yK1-expressing mice were bred with mice engineered by gene targeting to be deficient in mouse Ig production.

Initially, we examined the ability of yH1 to induce proper mouse B cell development and production of human Ig in a strain containing two functionally inactivated mouse heavy chain alleles (yH1;$\Delta J_H/\Delta J_H$). $\Delta J_H/\Delta J_H$ parental mice fail to rearrange their heavy chain genes, leading to a complete absence of mature B cells and a complete block in Ig production[18]. In yH1;$\Delta J_H/\Delta J_H$ mice, reconstitution of mature B cells (B220$^+$, hμ$^+$) was observed in bone marrow (Fig. 4*a*), spleen and blood (Fig. 4*e*) corresponding to 58%, 55% and 30% of the levels in wild-type mice, respectively. The majority of B220$^+$/hμ$^+$ cells in blood also expressed human delta (hδ) and all of the hδ$^+$ cells co-expressed hμ (data not shown), indicating proper expression and regulation of the human constant regions in these mice[19]. Remarkably, yH1;$\Delta J_H/\Delta J_H$ mice produced serum hμ at 350 μg ml^{-1}, a level 510-fold greater than parental 125A mice (Table 1*c*) and within 2–3-fold of normal human IgM serum levels. Hμ serum titres showed an age-dependent increase (not shown). These results demonstrate that yH1 can reconstitute B cell development in mice deficient in mouse heavy chains and direct the expression and assembly of high levels of hμ/mouse light chain antibodies.

The yH1;$\Delta J_H/\Delta J_H$ mice were further evaluated by analysing the orderly differentiation of bone marrow-derived B cells by flow cytometry using antibodies to the cell surface marker CD43, which defines early B cell subpopulations[20], in conjunction with antibodies to B220 and hμ. Bone marrow from $\Delta J_H/\Delta J_H$ mice contained no mature B cells (B220$^{low/+/high}$, CD43$^+$; R1, R2 populations) and a population (5.5%) of pro-B cells (B220^{high}, CD43$^+$; R3 population), comparable to wild-type mice (Fig. 4*a*). In contrast, yH1;$\Delta J_H/\Delta J_H$ bone marrow contained a nearly normal R1, R2 population (41%) of mature B cells; however, the size of the pro-B cell population (12.4%) was increased (Fig. 4*a*). Surface hμ was detected only in the B220$^+$, CD43$^-$ population of yH1;$\Delta J_H/\Delta J_H$ mice, similar to mμ in wild-type mice (Fig. 4*b*). Thus, while B cell development and Ig production are substantially restored in yH1;$\Delta J_H/\Delta J_H$ mice, the maturation of bone marrow-derived B cells may be somewhat less efficient than in normal mice.

To delineate this partial block in B cell maturation, we examined the surface expression of HSA and BP-1 in bone marrow to resolve the pro-B and pre-B cell populations[20]. Large, primarily CD43$^-$ populations of B220$^+$, HSA$^+$ cells and B220$^+$, BP-1$^-$ cells as well as a smaller CD43$^-$, B220$^+$, BP-1$^+$ cell population confirmed that B cell development is largely normal in these mice (Fig. 4*c,d*). However,

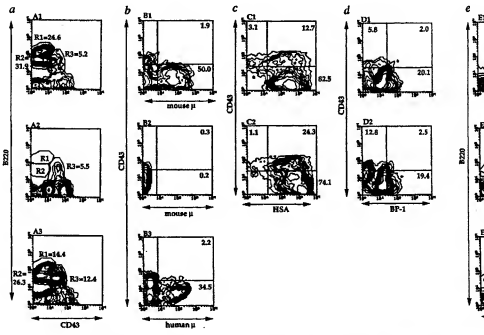

Fig. 4 Reconstitution of B cell development in ΔJ_H homozygous mutant mice by yH1 (125A strain) YAC. *a–d*, Bone marrow lymphocytes from normal control (129×B6: A1, B1, C1, D1), $\Delta J_H/\Delta J_H$ (A2, E1) or yH1;$\Delta J_H/\Delta J_H$ mice (A3, B2, B3, C2, D2) were assayed for surface expression of CD43 and B220 (*a*), or gated on B220$^+$ cells and assayed for surface expression of mμ and CD43 (B1, B2), hμ and CD43 (B3), HSA and CD43 (C), or BP-1 and CD43 (*d*). The bone marrow B220$^+$ subpopulations (R1, R2 and R3 (high (bright) or low (dull) levels of surface B220$^+$ are delineated)) are indicated with their respective percentages of positively stained cells. *e*, Peripheral blood (E1, E2) and spleen (*E3*) lymphocytes were assayed for hm and B220 in $\Delta J_H/\Delta J_H$ (E1) and in yH1;$\Delta J_H/\Delta J_H$ mice (E2, E3). The net percentage of positively-stained cells is shown in each quadrant. In the normal 129×B6 mouse, 64% of the cells in blood and spleen were B220$^+$. All animals used were 3 month old males.

increased populations of CD43$^+$, HSA$^+$ cells and CD43$^+$, BP-1$^-$ cells as well as smaller populations of CD43$^+$, HSA$^-$ cells and brighter CD43$^+$, HSA$^+$ cell populations indicated a small accumulation of pro-B cells, suggesting a less efficient pro-B cell to pre-B cell transition, the stage at which V to DJ joining occurs[20].

We next evaluated the ability of yH1 and yK1 together to restore B cell development and Ig production in a strain called Xenomouse, which also contains two functionally inactivated mouse heavy and kappa light chain alleles (yK1;yH1;$\Delta J_H/\Delta J_H$;$\Delta C_\gamma/\Delta C_\gamma$). In the $\Delta J_H/\Delta J_H\Delta C_\gamma/\Delta C_\kappa$ parental mouse, called DI (for double-inactivated), the expression of both mouse heavy and κ was blocked (manuscript in preparation). In contrast to DI mice which did not produce any mature B220$^+$ cells (Fig. 5*a1–c1*), mature B220$^+$ cells were present in Xenomouse at 10% of the level seen in the wild-type. Approximately half of these cells (43%) co-expressed hμ and hκ, while the remainder (57%) co-expressed hμ and mλ (Fig. 5*a2*, *b2*). No co-expression of mλ and hκ was detected, indicating that each light chain completely excluded the expression of the other (Fig. 5*c2*). Higher levels of fully human hμ/hκ antibodies (10 μg ml^{-1}) than hμ/mλ antibodies (3 μg ml^{-1}) were detected in Xenomouse. The level of hμ/hκ was at least several hundredfold higher than the level detected in the parental HuAb strain (9.2C;2B), confirming that inactivation of the mouse heavy and κ genes greatly increased levels of fully human antibodies. In addition, the relative level of B cells expressing only hμ and hκ in Xenomouse was 100–200-fold higher than in the HuAb strains from which antigen-specific human antibodies were obtained, suggesting the usefulness of Xenomouse in deriving fully human mAbs. Higher levels of hμ/hκ antibodies (200 μg ml^{-1}) were detected in another Xenomouse strain (8.2A; 2B; $\Delta J_H/\Delta J_H$;$\Delta C_\gamma/\Delta C_\kappa$). As HuAb strains producing higher levels of hμ/hκ are bred with DI mice to create additional Xenomouse strains, it is anticipated that higher levels of B cell reconstitution and antibody production will be attained.

Discussion

We have produced antigen-specific, fully human mAbs in mice and created mouse strains in which the majority of antibodies produced are fully human. The ability to derive antigen-specific human antibodies upon immunization of mice may be related to the diverse human adult-like repertoire observed following the introduction of large, intact germline segments of the human heavy and κ loci contained on YACs, in contrast to the abnormal repertoires seen in human Ig minigene-bearing mice[2–4]. While the human Ig repertoire of our YAC-containing mice are characterized by the V, D and J usage, length of N addition and CDR3 size observed for adult human B cells[12,13], earlier studies with minigenes revealed fetal-like, position-biased usage of D segments and abnormally small N addition and CDR3 size[3,4], and abnormal CDR3 sequences[2]. These differences, as well as the higher, less position-dependent expression levels noted for YAC-containing mice, may reflect the greater size, variable gene content, structural integrity upon integration, and/or presence of unidentified regulatory elements needed for optimal expression and proper regulation.

The possibility that human Ig genes compete inefficiently with mouse antibody genes by virtue of intrinsic activity or fewer V segments, led us to investigate human Ig expression in mice with inactivated mouse Ig genes. Together, human heavy and κ YACs restored B cell development in mice with inactivated mouse heavy and κ genes, and the majority of antibodies produced are fully human. Indeed, as half of the mature B cells of these mice

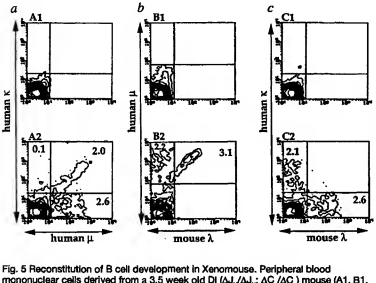

Fig. 5 Reconstitution of B cell development in Xenomouse. Peripheral blood mononuclear cells derived from a 3.5 week old DI ($\Delta\iota/\Delta\iota_J$; $\Delta C_\gamma/\Delta C_\gamma$) mouse (A1, B1, C1) or Xenomouse (9.2C; 28; $\Delta\iota_J/\Delta\iota_J$; $\Delta C_\gamma/\Delta C_\gamma$) (A2, B2, C2) were assayed for hμ and hκ (a), hμ and mλ (b) or hκ and mλ (c) as described in Methodology. The net percentage of positively stained cells is shown in each quadrant. All $hμ^+$, $hκ^-$ and $hμ^+$, $mλ^+$ cells were B220$^+$. In the normal mouse, 47% of peripheral blood mononuclear cells were B220$^+$. DI mice were generated by the breeding of homozygous $\Delta\iota_m$-mutant ($\Delta\iota_J/\Delta\iota_J$) mice[19] and homozygous ΔC_γ-mutant ($\Delta C_\gamma/\Delta C_\gamma$) mice. $\Delta C_\gamma/\Delta C_\gamma$ mice were derived by gene-targeted deletion of the mouse C_γ region (manuscript in preparation.)

express surface hμ and hκ, but not $mλ$, and the relative level of hμ/hκ B cells is at least 100-fold higher than in HuAb strains used to derive antigen-specific human antibodies, such mice should be extremely useful for obtaining human monoclonal antibodies upon immunization. The yH1 YAC induced the maturation of the growth-arrested B cell lineage in homozygous ΔJ_H-mutant mice, leading to the production of hμ levels approaching those in normal human serum. These results suggest that hμ can readily assemble with mouse B cell receptors, allowing the efficient development of mature, functional B cells. The analysis of bone marrow-derived B cells suggests that in yH1$\Delta J_H/\Delta J_H$ mice the progression of late pro-B to pre-B cells is somewhat less efficient than in normal mice, a point at which V-DJ rearrangement is initiated[20]. The limited number of V genes in yH1 may result in a lower frequency of rearrangement and thus incomplete B cell development (analysis in progress), suggesting the value of a greater number of human V genes to support more complete B cell maturation and the generation of even more complex repertoires.

The ability to produce a diverse repertoire of fully human monoclonal antibodies may have significant application to human therapy. Unlike humanized mouse antibodies which contain a significant number of residues from murine hypervariable regions, fully human antibodies may be less immunogenic, and thus more suited for repeated administration, as they would present only minor idiotypic variations from any given patient. Such mice lack immunological tolerance to and thus readily yield antibodies to human proteins, which may constitute an important class of therapeutic targets. The introduction of larger portions of the human heavy and light chain loci may ultimately yield strains of mice capable of recapitulating the full repertoire characteristic of the human humoral response to infection or immunization. This strategy of introducing large segments of the human genome into mice coupled with inactivation of the corresponding mouse genes may also have applicability to the investigation of other complex or uncharacterized loci.

Note added in proof: Hybridomas producing human antibodies against a human protein, IgE antibody, have been generated.

Methodology

Generation and DNA analysis of yH1- and yK1-containing ES cells and mice. yH1 (240 kb) and yK1 (195 kb) were identified from the Washington U. human-YAC library DNA using V_H PCR primers and a C_μ probe, respectively (M.J.M. *et al.*, manuscript in preparation). yH1- and yK1-containing yeast were fused with E14.TG3B1 cells as described[2] and HAT-resistant colonies were expanded for analysis. Probes used: D (detecting three expected *Hind*III bands, 9.7 kb, 8.5 kb and 7.0 kb (as a doublet), of D1–D4 family segments), $Cμ$, $Cδ$, J_H, V_{H1}, V_J and V_{H6} as described in ref. 21; V_H[21], C_γ (ATCC, 59173), J[22], $B1$[23], $B2$[23], $B3$[23], mC_H[22] and yeast Ty^1. The number of integrated YACs per ES cell was determined by densitometric measurements of human Ig hybridization signals, normalized to those of mouse C_κ probe, and compared to the signal intensity of YACs in yeast. An equivalent hybridization signal should be obtained from a single-copy sequence in 20 ng of haploid yeast DNA and in 10 μg of diploid mammalian DNA.

Chimaeric mice were generated by microinjection of ES cells into C57Bl/6 blastocysts. HuAb offspring were identified by PCR analysis of tail DNA (30 cycles of 1 min at 94 °C; 2 min at 60 °C; 3 min at 72 °C) using human-specific primers for D: (DXPAs 5'-GCAATTACTAGTATAGACCCGAGTGTCCCC-3'; and DXPBs 5'-GCAATGGTCGACGGTTTTTGATGGACTCTG-3'; 230 bp fragment), and J_H: (J$_H$0: 5'-CTCGAAAAGGGAGTTGAGCT TCAGCAGCTG-3'; and J$_H$3–5: 5'-GATACAATGGCACTAAAATC TCACG-3'; 860 bp fragment) and control primers for the mouse γ interferon receptor (IFN-γR) (5'-TTGGATTCTGGTG GTTGCTC-3' and 5'-GACCTATTTGTGCATTGGAAGC-3'; 550 bp fragment). The size markers (M) are a 1 kb DNA ladder (Gibco/BRL).

Flow cytometry analysis. Peripheral blood, spleen and bone marrow lymphocytes obtained from 4–12 week old transgenic or control mice were purified on Lympholyte M (Accurate) and treated with purified Fc γ II receptor (Pharmingen, 01241D) to block non-specific binding to Fc receptors, stained with antibodies and analysed on a FACScan (Becton Dickinson, LYSIS II software). Antibodies used: CyChrome anti-B220 (Pharmingen, 01124A); fluorescein isothiocyanate (FITC) anti-hIgM (Pharmingen, 08074D); phycoerythrin (PE) anti-mIgM^a (Pharmingen, 05095B) and PE anti-mIgM^b (Pharmingen, 05105B); biotin anti-hδ (Southern Biotechnology Association, 9030-08); biotin anti-hκ (Pharmingen, 08172D); FITC anti-mλ (Pharmingen, 02174D); FITC anti-CD43 (Pharmingen, 01604D); PE anti-CD4 (Pharmingen, 01605B); biotin anti-6C3/BP-1 (Pharmingen, 01282D); PE anti-HSA (Pharmingen, 01575A). PE-streptavidin (Pharmingen, 13025D) was used to detect biotinylated antibodies.

To assay hκ versus mκ expression in yK1 transgenic mice, mouse peripheral blood lymphocytes were first incubated with rat IgG (Pierce, 31233) then stained with PE-conjugated goat anti-mκ (Southern Biotechnology Association, 1050-09), washed, incubated with mouse IgG (Pierce, 31204), then stained with FITC-conjugated mouse anti-hκ (Pharmingen, 08174D) and CyChrome-conjugated anti-B220.

ELISA assays. Sera were obtained from 4–12 wk old transgenic or chimaeric (containing 40–90% of ES cell-derived B cells) mice. Human serum μ was assayed using mouse monoclonal anti-hμ (AMAC, Clone Af6) immobilized on Nunc Immuno plates (Maxisorp F96) and detected with biotinylated goat anti-hμ (Caltag, preabsorbed with normal mouse serum to lower background due to cross reactivity). Similarly, hκ was assayed using goat anti-hκ (Vector) to capture and detected with biotinylated goat anti-hκ (Vector). The standard used to determine hμ concentrations was hIgM (Sigma, 1-

8260) shown to be equivalent to a chimaeric hIgM/mλ antibody (Serotec, MCA 446). The standard used to determine hκ concentrations was hIgG$_1$/hκ (Sigma, I-3889). Hμ/hκ antibodies were detected in serum by ELISA using mouse monoclonal anti-hμ (AMAC, Clone A#6) to capture and detected with biotinylated goat anti-hκ (Vector) using hIgM (Sigma, I-8260) as a standard. Tet C-specific antibodies (polyclonal or monoclonal) were assayed by coating plates (see above) with 100 ng tet C (Boehringer Mannheim, 1348655)/well, incubating with serial dilutions of the appropriate serum and then detecting with either biotinylated goat anti-hμ (Caltag, see above) or biotinylated goat anti-hκ (Vector). Human monoclonal antibodies were assayed the same as hμ/hκ antibodies above. Biotinylated antibodies were detected using ABC-HRP (Vector, PK-4000). Absorbance at A490 was measured using a UVmax spectrophotometer.

Immunization of mice and generation of hybridomas. Mice were immunized 4 times at about 2 wk intervals with tet C (Boehringer Mannheim 1348655) 50 μg/injection, in Freund's Complete Adjuvent (primary injection) or Freund's Incomplete Adjuvent (subsequent boosts) subcutaneously. The mice were bled 4 days after final boost and titered for human anti-tet C antibodies by plate ELISA. Serially diluted samples were incubated with 100 ng tet C bound/well of Nunc Immunopalate. The human antibody chains were then detected using the biotinylated anti-hμ and hκ antibodies (see "ELISA assays"). To generate mouse hybridomas, splenocytes from immunized mice were fused with nonproducer P3X63-Ag8.653 myeloma cells, 4–5 days following the final boost, using 50% PEG 4000 (Boehringer Mannheim). After 10–14 days the supernatants from hybrids growing in HAT-selected medium were screened for the presence of fully human antibodies and for tet C specificity as described above.

Repertoire of human Ig transcripts expressed in transgenic mice. poly(A)$^+$ mRNA was isolated from a yH1 spleen (strain 2B) or a yK1 spleen (strain 8.2A) transgenic mouse using a FastTrack kit (Invitrogen). Human μ-specific mRNAs amplified using a 5' Amplifinder RACE kit (Clontech), using oligonucleotide hμP1 (5'–TTTTCTTTGTTGCCGTTGGGGTGC–3') for reverse transcription and hμP2 (5'–GGGAAGCCCCGGGTGCTGCTGATG–3') for amplification. Human κ-specific cDNAs were amplified using hKP1 (5'-CTCTGTGACACTCTCCTGGGAGTT-3') for reverse transcription and hKP2 (5'–ACCCGATTGGAGGGCGTT-ATCCAC–3') for amplification. PCR products were cloned into pCRII using a TA Cloning kit (Invitrogen) and their sequences determined by double-stranded dideoxynucleotide sequencing. For hybridization experiments, individual colonies were picked onto multiple gridded LB-amp plates and grown at 37 °C. Colonies were transferred to Genescreen (DuPont) and lysed *in situ*[8] to yield replicas of the ordered grid. DNAs immobilized on filters were probed with ^{32}P-labelled V_κ or C_H1 fragments for yH1 cDNAs or with V_κ fragments for yK1 cDNAs (see above). ^{32}P-end labelled J_H- or J_κ-specific oligonucleotides were hybridized to DNAs on filters in 1% bovine serum albumin, 1.25 mM EDTA, 0.5 M $NaPO_4$ buffer, pH 7.2, and 7.1% SDS at 58 °C except J_H6 which was hybridized at 30 °C. Filters were washed three times in 6x SSC for 3 min at room temperature then washed once in 6x SSC for 3 min at 34 °C with the exceptions that J_H6-probed filters were washed at 30 °C and $J_\kappa5$-probed filters were washed at 42 °C. J_H-specific oligonucleotides were: J_H1: 5'–ACTTCCAGCACTGGC–3'; J_H2: 5'–ACTTCGATCTCT GG–3'; J_H3: 5'–TTTTGATATCTGGGGG–3'; J_H4: 5'–TTTGACTACT– GGGGC–3' J_H5: 5'–TTCGACCCCTGGGGC–3' J_H6: 5'–TACTA– CTACTACTAC–3'. J_κ-specific oligonucleotides were: $J_\kappa1$: 5'–AGGTGGAAATCAAAC–3' $J_\kappa2$: 5'–TTTGGCCAGGGGACC–3' $J_\kappa3$: 5'–TTCGGCCCTGGGACC–3' $J_\kappa4$: 5'–TTTCGGCGGAGGGAC–3' $J_\kappa5$: 5'–AGGGACACGACTGGA–3'.

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The human immunoglobulin loci introduced into mice: V (D) and J gene segment usage similar to that of adult humans

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Variable gene segments of the human immunoglobulin loci are represented in the human peripheral repertoire at different frequencies. XenoMouse™ strains contain approximately 2 megabases of the human immunoglobulin heavy and kappa light chain loci that functionally recapitulate the human humoral immune system. Analysis of human antibody transcripts from XenoMouse spleens and lymph nodes revealed that V, D and J gene segment utilization from these unimmunized animals were nearly identical to the gene segment utilization reported for humans with extensive antigenic histories.

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1 Introduction

The utilization of Ig gene segments comprising the human humoral repertoire is not random [1–6]. Specific gene segments are over-represented while other gene segments are significantly under-represented. In general, the distribution of H chain variable gene segment families reflects their germ line complexity, *i.e.* V_H3, the largest family, is found the most often, followed by V_H4 and V_H1 [4, 5, 7, 8]. However, not all the V_H gene segments within a family are represented equally [6, 9, 10]. In fact, relatively few gene segments from a V_H gene family may constitute the majority of the observed repertoire for that family. Non-random utilization of V_H gene segments has been confirmed in multiple studies employing a variety of molecular approaches, each with inherent biases, and representing repertoires of different individuals, each with unique genetic polymorphisms and distinct antigenic histories.

While initial studies focused on characterizing the gene segment utilization of the human antibody repertoire, recent studies have focused on the mechanisms of selection that shape the development of the human Ig repertoire. Antigenic history, in the context of both positive and negative selection, has been cited as an important factor in the generation of mature B cells [11–13]. B cells from different organs or lineages (fetal, intestinal, B1, B2) may contain distinct repertoires that serve specialized functions [14–17]. Analyses performed on pre-B cells suggest that development in the bone marrow and pairing with the surrogate L chain shapes the primary repertoire [18–21]. How much of the observed human antibody repertoire, including variable gene segment bias, is intrinsic to the Ig locus and the process of B cell development versus exposure to external antigens has not been determined.

Recently we have demonstrated the functional transplantation of a majority of the human Ig H and kappa L chain loci into the germ line of mice that have been inactivated for endogenous antibody production [22, 23]. These transgenic animals, referred to as XenoMouse™ strains, contain, in substantially germ-line configuration, over 1.0 megabase of the human IgH locus (66 V_H gene segments) and approximately 800 kb of the human Ig kappa locus (32 kappa chain variable gene segments). XenoMouse strains functionally recapitulate the human antibody response including a vast repertoire of high-affinity, somatically hypermutated human antibodies.

As XenoMouse strains contain the majority of the human Ig H and kappa chain loci, essentially in germ-line configuration, we were able to perform experiments on the human humoral response that are not possible in human subjects. For the first time, it was possible to determine

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Abbreviations: RT: Reverse transcription **DIR:** D genes with irregular recombination signals **RSS:** Recombination sequence signals

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the V gene segment utilization of an unimmunized and specific pathogen-free human antibody repertoire. Gene segment utilization of the V, D and J gene segments indicates that XenoMouse strains faithfully reproduce the human adult antibody repertoire.

2 Results

2.1 General remarks

To characterize the utilization of human V gene segments in XenoMouse strains, human antibody transcripts were isolated from adult lymph node and spleen. Following RNA isolation and the generation of cDNA, different V gene-specific oligonucleotides [24] were used in PCR amplification reactions. Mixed primers, representing all the V gene families or primers to a single V gene family were used to generate products that were subsequently cloned. Sequence analysis of the antibody transcripts was performed to identify the relative frequency at which a specific gene segment was found in the repertoire.

Since the XenoMouse strains in this study contain only a single allele of the human H chain locus and a single copy of the human kappa chain locus, these animals are functionally hemizygous. The result is that all H chain transcripts isolated from peripheral secondary immune organs such as the lymph node and spleen represent in-frame functional rearrangements. Although XenoMouse strains have a functional mouse lambda locus, mouse lambda-positive B cells contribute to less than 15 % of the mature B cells and as a result the vast majority of human kappa transcripts (greater than 90 %) represent in-frame and productive rearrangements [23].

2.2 Utilization of V gene segments in XenoMouse human H chain transcripts

The entire human H chain locus that has been introduced into XenoMouse strains is accessible to V(D)J rearrangement. Sequence analysis of the 120 H chain IgM transcripts in this report as well as the sequence analysis of over 50 hybridomas generated from XenoMouse strains (data not shown) has identified 24 of the 34 functional human V_H gene segments present in XenoMouse. There does not appear to be any positional bias in V_H gene segment utilization. V_H segments located both distally, nearly 1 megabase away (4–61), as well as the V_H gene segment most proximal to the J_H locus (6–1) are utilized. The V_H gene segments yet to be detected in XenoMouse H chain transcripts represent the same gene segments that are under-represented in the human repertoire (see below).

Table 1. V_H diversity

	Number utilized/number present in genome[a]	
Family	XenoMouse	Human PBL[b]
V_H1	4/8 (50 %)	6/9 (67 %)
V_H2	1/2 (50 %)	2/3 (67 %)
V_H3	10/15 (67 %)	13/22 (59 %)
V_H4	7/7 (100 %)	7/10 (70 %)
V_H5	1/1 (100 %)	1/2 (50 %)
V_H6	1/1 (100 %)	1/1 (100 %)
V_H7	0	0
Total clones	24/34 (71 %)	30/47 (64 %)

a) Numbers in parentheses indicates percentage of available family members observed in the expressed repertoire.
b) Brezinschek et al. [5].

Similar to H chain transcripts from human PBL, XenoMouse strains show a biased usage of V_H gene segments within a family (Table 1). For example, only 13 of the potentially 22 functional V_H3 family members (59 %) are actually observed in the human peripheral adult human repertoire. In XenoMouse strains only 10 of the 15 functional V_H3 gene segments present on the transgene (67 %) are found expressed in the repertoire. Similarly XenoMouse strains use 4 out of the 8 functional V_H1 family members (50 %), which compares favorably with the 6 out of 9 (67 %) found in human PBL. This pattern was consistent throughout the V_H families and demonstrated that the percentage of functional V_H segments for a given family that is actually present in the XenoMouse repertoire corresponds closely to that reported in humans (Table 1).

The V_H3 and V_H4 families represent the most frequently observed gene segments in the human adult repertoire. To ascertain the frequency of each V_H family in the peripheral repertoire of XenoMouse strains, a human V_H consensus primer was used in conjunction with a $C\mu$-specific primer to generate PCR products corresponding to human IgM transcripts (see Sect. 4.2). Forty-seven sequences analyzed in this experiment showed a non-random frequency of utilization, with V_H3 and V_H4 families representing 62 % and 25 %, respectively, of the generated transcripts. This is very similar to the 56 % and 20 % frequencies typically found for these V_H families in the human repertoire (Table 2).

Table 2. V_H Family usage[a]

Family	XenoMouse	Human PBL[b]
V_H1	1 (2 %)	9 (13 %)
V_H2	0[c]	3 (4 %)
V_H3	29 (62 %)	40 (56 %)
V_H4	12 (25 %)	14 (20 %)
V_H5	5 (11 %)	4 (6 %)
V_H6	0[c]	1 (1 %)
V_H7	0	0
Total clones	47	71

a) Numbers in parentheses indicate percentage of all genes detected that were members of each family.
b) Brezinschek et al. [5].
c) Gene segments from the V_H2 and V_H6 family were not observed in the analysis of these 47 sequences but were identified as functional in the analysis of hybridomas and fetal tissues.

Bias in gene segment utilization is even observed within a V_H family; specific V_H gene segments are characteristically over-represented while other gene segments are under-represented in the repertoire of human PBL. For example, V_H gene segments 3–23 and 4–34 represent over a quarter of the observed gene segments derived from the V_H3 and V_H4 families, respectively. To determine if the V_H gene segment utilization in XenoMouse strains is the same as that observed in humans, we examined the utilization of specific V_H gene segments within a V_H family. Reverse transcription (RT)-PCR was performed separately with V_H3 and V_H4 family-specific primers (see Figs. 1 and 2). Seventeen sequences were analyzed using the V_H3 family-specific primer set. As shown in Fig. 1, despite a relatively small sample size, the pattern of V_H segment utilization for the V_H3 family in the XenoMouse repertoire is strikingly similar to that reported for the human repertoire. The frequency of a specific V_H3 gene segment ranges from 0 % to close to 30 %. Notably, the V_H gene segments (3–23, 3–30 and 3–33) that are over-represented in the human peripheral repertoire are also over-represented in the XenoMouse repertoire. In both the human and XenoMouse repertoire V_H3 gene segments 3–20, 3–43, and 3–64 are absent. V_H gene segments 3–13, 3–21, and 3–48 are observed infrequently in both the XenoMouse and human repertoires. Although these latter three V_H3 gene segments were not identified in the set of 17 sequences analyzed in this experiment, they were identified in XenoMouse transcripts from other experiments (data not shown), confirming that they are functional but simply under-represented. A similar analy-

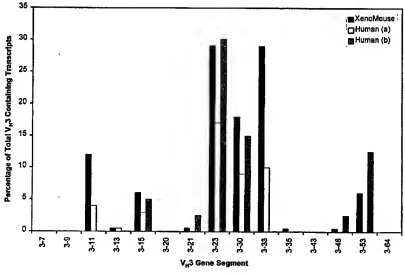

Fig. 1. Relative utilization of V_H3 gene segments. The percentage of specific V_H3 gene segments found in the analysis of 17 XenoMouse-derived transcripts obtained by PCR with a V_H3 family-specific primer is shown. The utilization of V_H3 gene segments in human antibody repertoires is derived from (a) Suzuki et al. [6] and (b) Brezinschek et al. [5]. XenoMouse V_H3 gene segments 3–13, 3–21, 3–35 and 3–48 represented as less than 1 % were not observed in our analysis of 17 V_H3 transcripts but were identified as being functional in other experiments.

sis of 17 sequences generated with a V_H4 family-specific PCR primer set is shown in Fig. 2. A bias in gene segment usage within the V_H4 family was also observed. The same V_H4 gene segments 4–4, 4–31, 4–59 and 4–34 over-represented in XenoMouse strains are also over-represented in the human repertoire (Fig. 2). The V_H4 gene segments 4–28, 4–39, and 4–61 were under-represented in both the XenoMouse and human repertoires.

The results of our analysis of V_H3 and V_H4 gene segment utilization demonstrate that not only are the same V_H gene segments utilized but that they are utilized very much to the same degree. As a result, the V_H gene segment representation in the XenoMouse repertoire appears to be substantially the same as that observed in humans.

2.3 Utilization of D and J_H gene segments in XenoMouse antibody transcripts

In addition to V gene segments, D and J_H gene segment utilization also contributes to the generation of a diverse human H chain repertoire. It is well recognized that the analysis of D segment usage is often difficult because only a small portion of the D segment may be incorporated into the V(D)J junction. Often sequences as short as five nucleotides or less can be identified that have homology to a known D segment. Analysis of 120 XenoMouse transcripts identified 113 with greater than or equal to 5 bases of D sequence homology. Of the 7 transcripts with fewer than 5 bases of homology to a D element, only 2 transcripts could not be assigned to a D segment. Using the nomenclature of Corbett et al. [25] we have identified 19 of the 23 functionally distinct D segments of the human D locus in H chain transcripts derived from XenoMouse strains. The D elements were utilized throughout the D locus and did not show any positional bias in their utilization (Fig. 3). In addition to the conventional D gene segments, 18 % of XenoMouse transcripts had homology that aligned with D genes with irregular recombination signals (DIR) sequences [26].

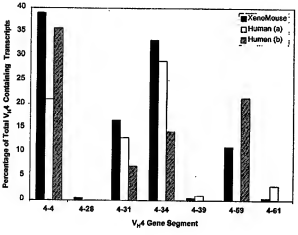

Fig. 2. Relative utilization of V_H4 gene segments. The percentage of specific V_H4 gene segments found in the analysis of 17 XenoMouse-derived transcripts obtained by PCR with a V_H4 family-specific primer is shown. The utilization of V_H4 gene segments in human antibody repertoires is derived from (a) Suzuki et al. [6] and (b) Brezinschek et al. [5]. XenoMouse V_H4 gene segments 4–28, 4–39 and 4–61 represented as less than 1 % were not observed in our analysis of 17 V_H4 transcripts but were identified as being functional in other experiments.

The J_H gene segment utilization in XenoMouse strains and humans is biased to J_H4 and J_H6. Table 3 shows the frequency of human J_H gene segment usage in 120 XenoMouse-derived H chain transcripts compared to the utilization observed in adult PBL. The most frequently observed J_H segments in the XenoMouse repertoire are J_H4 (46 %) and J_H6 (39 %). Gene segments J_H5 and J_H2 are observed at a frequencies very similar to those observed in the human repertoire, *i.* e. 5 % and 3 %, respectively. The J_H1 utilization in humans is approximately 1 %. A similar frequency in XenoMouse strains may account for the absence of the J_H1 gene segment in the 120 transcripts analyzed. The utilization of J_H gene segments observed in XenoMouse strains closely parallels that reported for human repertoires in every respect (Table 3).

3 Discussion

Our analysis of the human antibody repertoire in XenoMouse strains confirms that the utilization of human Ig V_H gene segments is not random and demonstrates that the human antibody H chain repertoires of XenoMouse strains and human PBL are remarkably similar (Figs. 1 and 2). The precision with which the repertoire of XenoMouse strains has recapitulated the human repertoire is exemplified by the selection of specific V_H3 and specific V_H4 gene family members that are over-represented and under-represented in both the XenoMouse and human repertoires. D segment utilization, including the use of DIR elements unique to primates, D-D fusions and D inversions (data not shown), as well as the preferential utilization of J_H gene segments J_H4 and J_H6 are all observed at frequencies similar to those reported in human repertoires. The human V_κ gene segment utilization in the XenoMouse repertoire is also similar to that

Table 3. Human J_H gene segment utilization

J_H Gene	XenoMouse	Human PBL			
		a)		b)	
J_H1	0 (0 %)	1	(1 %)	1	(1 %)
J_H2	4 (3 %)	3	(4 %)	0	(0 %)
J_H3	9 (7 %)	6	(9 %)	9	(9 %)
J_H4	55 (46 %)	29	(41 %)	52	(52 %)
J_H5	6 (5 %)	5	(7 %)	15	(15 %)
J_H6	47 (39 %	27	(38 %)	22	(22 %)
Total[c]	121	71		99	

a) Brezinschek et al. [5].
b) Yamada et al. [3].
c) Total number of transcripts analyzed.

observed for the human repertoire (data not shown). The $V_\kappa I$ and $V_\kappa III$ families are the most frequently expressed V_κ gene segments and specific V_κ gene segments like A27 and 012 are also abundantly expressed in both the human and the XenoMouse repertoires. The relative utilization of specific $V_\kappa I$ family gene segments in the human kappa repertoire of XenoMouse also parallels that observed in humans.

In conclusion, XenoMouse, with its limited antigenic history, is functioning with apparently the same intrinsic bias in segment utilization as the human repertoire. The basis of the biased usage is still not clear given that V gene segments with identical recombination sequences show dramatic differences in their representation in the human repertoire. The V_H gene segments 4-4 and 4-28 have identical recombination signals [27, 28] yet are observed at dramatically different frequencies in the human repertoire (Fig. 2). DNA sequences flanking the recombination sequence signals (RSS) as well as the roles of enhancers and transcription have all been reported to affect recombination efficiency. Cis-acting elements are also implicitly involved in determining chromatin accessibility that in turn influences targeting of the recombinase to the proper RSS. V(D)J recombination is also very tightly regulated during B cell development; D–J recombination occurs prior to V–DJ recombination that is subsequently followed by recombination of the L chain loci. Recent studies utilizing *in vitro* assays have demonstrated that chromatin accessibility of the recombinase is programmed in different cell lineages [29, 30]. It remains to be determined if V gene segment utilization is also dictated by chromosome accessibility. Other studies have noted differences between individual repertoires and have attributed these differences to gene segment polymorphisms [6, 9, 31]. It remains to be seen whether these polymorphic differences observed in humans result in repertoires that are responsible for autoimmune disease or susceptibility to infection or cancer.

XenoMouse strains represent a significant improvement from earlier transgenic mice carrying a limited number of human Ig genes not only because of the immense size of the loci present in these animals but also because the human loci are in germ-line configuration. As a result, XenoMouse strains contain a human humoral back-

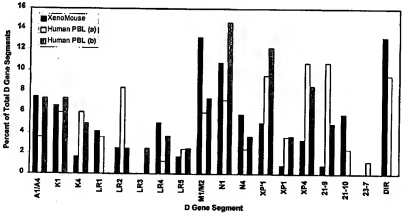

Fig. 3. Usage of D segments in XenoMouse strains and human repertoires. The distribution of D segments in human H chain transcripts based on the sequence analysis of 120 XenoMouse-derived transcripts is shown. The utilization of D elements observed in human antibodies derived from (a) Yamada et al. [3] and (b) Brezinschek et al. [5].

ground and represent a model system that allows for studies not possible in humans. XenoMouse strains can be exploited to determine the significance of the restricted V gene segment utilization that has been observed in human malignancy, autoimmune diseases and responses to bacterial pathogens. They may also be utilized to study V gene segment utilization in response to new vaccines in order to predict their utility in generating the appropriate human immune response. In addition, XenoMouse strains can potentially be used as surrogates for humans to screen humanized antibodies for immunogenicity.

4 Materials and methods

4.1 XenoMouse strains

The generation of XenoMouse strains has been previously described [22, 23]. The XenoMouse strains in this study were functionally hemizygous for the human H chain and human kappa L chain loci. XenoMouse strains are maintained in an SPF and full barrier configured animal facility. Unimmuized mice were used in all experiments.

4.2 RT-PCR and primers

Lymph nodes (approximately 20) and spleens from 4- to 8-week-old XenoMice were isolated and processed according to the manufacturer's instructions using the Micro-Fast Track and Fast-Track 2.0 kits for the isolation of poly (A+) RNA (Invitrogen). The PCR amplification protocol and primers have been previously described [22, 24]. V_H family-specific primers were pooled or used individually as indicated.

4.3 Sequencing and analysis

Sequencing was performed using 4.75 % acrylamide gels, Prism dye terminator sequencing kits and the 373 DNA sequencer (Applied Biosystems). Sequences were analyzed with MacVector and GeneWorks software. The Vbase human antibody database was used for sequence alignments and gene segment identifications (Tomlinson et al., MRC Centre for Protein Engineering). DNA sequences were aligned sequentially to first identify the V_H segment and then the J_H segment. The intervening sequence that had not homology with either the V_H or J_H segments was then aligned against a database of human D segments in V base and the best alignment was identified.

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and bats combined were not significantly different (Fig. 3; echolocators gradient = 0.7932, non-echolocators gradient = 0.8, analysis of covariance (ANCOVA) gradients $F = 0.034$, $p = 0.954$, d.f. 1,20). Thus, it is clear that the estimates of flight cost in echolocating bats are not significantly greater than those for non-echolocating bats.

The absence of an energy cost for echolocation for flying animals may explain why echolocation systems are widespread in the Microchiroptera, but have evolved in very few terrestrial animals. Furthermore the high cost for terrestrial animals[3] may explain why those systems that have evolved in terrestrial mammals involve only very weak short-range pulses[9]. Two alternative hypotheses may also explain the paucity of, and low intensity of, terrestrial echolocation systems. First, the echolocation pulses may reveal the whereabouts of the emitter to potential prey and predators. Alternatively, in a complex and cluttered terrestrial environment the emitter may be confused by strong reflections from very close large objects. Our data strongly support the energy cost hypothesis but cannot rule out these alternative hypotheses in the evolution of echolocation.

The close link between flight and reduced costs for echolocation raises the issues of why echolocation has evolved so infrequently amongst the birds and Megachiroptera, and why, when it has evolved in these animals, it is primarily used for gross navigation rather than prey detection. We suggest that the paucity of echolocation systems amongst these groups reflects a phylogenetic constraint on the development of the processing capacity for complex echolocation signals in animals which are already evolutionarily committed to a visual system. Vision is clearly the dominant system amongst birds. Nocturnal birds have larger cortex areas devoted to processing olfactory stimuli than diurnal birds[10]. As total brain size remains unaffected this suggests there is a trade off in the processing capacity allocated to the various senses. This may have prevented any species from making a complete evolutionary change from one system (visual) to another (echolocation) because the intermediate steps would be selectively inferior to either of the pure systems. The recent suggestion that the megachiroptera have a primate ancestry[11] is consistent with this interpretation because primates also have well-developed vision. □

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A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene

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Of the various classes of antibodies that B lymphocytes can produce, class M (IgM) is the first to be expressed on the membrane of the developing cells. Pre-B cells, the precursors of B-lymphocytes, produce the heavy chain of IgM (μ chain), but not light chains[1]. Recent data suggest that pre-B cells express μ chains on the membrane together with the 'surrogate' light chains λ5 and VpreB (refs 2–7). This complex could control pre-B-cell differentiation, in particular the rearrangement of the light-chain genes[8]. We have now assessed the importance of the membrane form of the μ chain in B-cell development by generating mice lacking this chain. We disrupted one of the membrane exons of the gene encoding the μ-chain constant region by gene targeting[9] in mouse embryonic stem cells[10]. From these cells we derived mice heterozygous or homozygous for the mutation. B-cell development in the heterozygous mice seemed to be normal, but in homozygous animals B cells were absent, their development already being arrested at the stage of pre-B-cell maturation.

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The vector used for the disruption of one of the membrane exons (μM) (Fig. 1*A*) contains 9 kilobases (kb) of genomic DNA spanning exons 1 and 2 of μM and the first three exons of the constant (C) region of the δ gene. Close to the 5′ boundary of the first exon of μM we introduced a translational stop codon and a *Sal*I site into which a neomycin-resistance gene (*neo*^{r}) cassette[11] was inserted. At the 3′ end of the genomic sequence we placed the herpes simplex virus thymidine kinase gene to permit selection against random integration[12].

Cells of the embryonic stem cell clone D3[13] were transfected with the linearized vector by electroporation and selected by G418 and gancyclovir on feeder layers of STO fibroblasts[14]. Surviving colonies were screened for homologous recombinants using the polymerase chain reaction (PCR) (see legend to Fig. 1). PCR-positive clones were expanded and their identification as homologous recombinants verified by Southern blotting (Fig. 1*B*). From 3.4×10^7 transfected embryonic stem cells 1,870 colonies were resistant to G418 (determined by control plates), 230 were resistant to both G418 and gancyclovir and in six clones one of the two $C\mu$ genes in the genome was modified by homologous recombination with the vector without random integration. Thus, the frequency of gene targeting was 1/38 G418^r+GANC^r (G418 and gancyclovir-resistant respectively) colonies, which corresponds to 1/312 G418^r colonies or $1/5.7 \times 10^6$ transfected cells.

The mutated clones were injected into blastocysts from C57BL/6 mice to generate chimaeric animals. As the D3 line is derived from an agouti mouse (strain 129/Sv), chimaeric mice could be identified by coat colour. Ten male chimaeras derived from four different mutated clones were mated to C57BL/6 females. One of these chimaeras, derived from clone 210, transmitted embryonic stem-derived chromosomes into the germ line as judged by the production of agouti offspring at a

frequency of 1:6. Three of ten such agouti animals contained the mutated allele as shown by Southern blotting (Fig. 2*b*). These animals permitted a first analysis of the effect of the μM mutation, designated μMT, on B-cell development. Flow cytometric analysis of peripheral blood B cells (identified by the CD45R(B220) surface antigen[15]) demonstrated normal levels of such cells in the heterozygous animals as compared with (C57BL/6 × 129/Sv)F_1 controls, but all cells expressed IgM of C57BL/6 origin, that is of the *b* allotype. This is in contrast to the situation in the F_1 controls where half of the cells express IgM^b and half IgM^a (of 129/Sv origin; Fig. 3). Thus, the μMT mutation is indeed correctly targeted to the $C\mu$ locus, preventing the expression of the membrane form of the μ chain from the targeted allele.

Studies with Abelson virus-transformed pre-B cell lines[8] and immunoglobulin transgenic mice[16] have suggested that the μ chain of membrane-bound IgM (the μ_m chain) plays a crucial role in mediating allelic exclusion, that is, the expression in a single B cell, of only one allele at the heavy-chain loci. The heterozygous mice depicted in Fig. 3 allow this hypothesis to be tested directly: if true, then productive rearrangement of the targeted locus should not preclude productive rearrangement on the other homologue. We indeed found that a small percentage of the B cells in these animals carry a-allotype antibodies of class D on the surface and that low levels of IgM^a can be detected in the serum (data not shown).

The effect of the μMT mutation on the development of B lineage cells can best be studied in homozygous mutant mice. Such animals (14.2 and 14.3; Fig. 2*c*) were generated by intercrossing animals heterozygous for the mutation (G5 end G7; Fig. 2*b*). At the age of 4 weeks, cells from blood, spleen and bone marrow of $\mu MT/\mu MT$ animals were analysed for the presence of B lineage cells by flow cytometry. Cells from heterozygous ($\mu MT/+$) and normal (+/+) littermates served as controls (Fig. 4). As expected, peripheral blood lymphocytes (Fig. 4*a*) and spleen cells (Fig. 4*b*) of the homozygous mutant mice lacked $CD45R(B220)^{bright}$ cells, indicating the absence of mature B cells. We detected neither cells expressing IgM or IgD

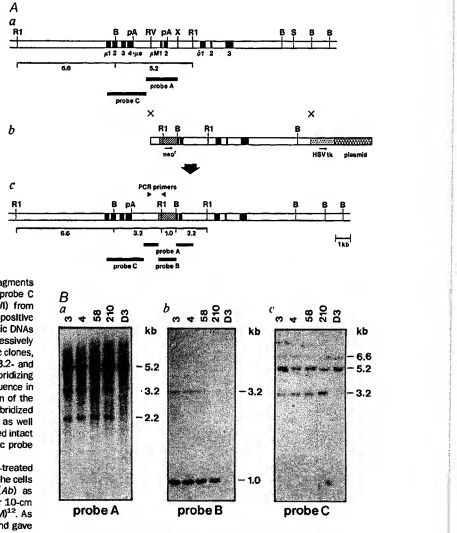

FIG. 1 *A*, Strategy for the disruption of the membrane exon of the $C\mu$ gene. *a*, Genomic structure of the *a* allele of the $C\mu$–$C\delta$ gene locus. Exons are represented by black boxes. pA, polyadenylation sites for the secretory (μ_s) and the membrane (μ_m) form of the μchain[17]. The lengths of diagnostic restriction fragments and location of probes used for Southern blot analysis are shown. RI, *Eco*RI; B, *Bam*HI; RV, *Eco*RV; X, *Xho*I, S, *Sal*I; *b*, Targeting vector containing a 9-kb *Eco*RV–*Sal*I fragment of the $C\mu$–$C\delta$ locus. The third codon of membrane exon 1 ($\mu M1$ in *a*) was changed to TAG followed by an insertion of TCGAC to create a *Sal*I site for site directed mutagencsis. Five base pairs (bp) downstream of the *Sal*I site, 7 bp were accidentally deleted. An *Xho*I–*Sal*I fragment (*neo*^{r}) of pMC1Neo-polyA[13](PMC1POLA, STRATAGENE) was inserted into the new *Sal*I site. The *Eco*RV–*Sal*I fragment carrying the *neo*^{r} gene was inserted into an *Xho*I site of pIC19R/MC1–Tk[12] (gift of K. Thomas, S. Mansour and M. Capecchi) which contains the HSV *tk* genc. The vector was linearized by *Cla*I before transfection. *c*, Predicted structure of the targeted locus. Triangles indicate the primers used for PCR assays. The sequence of the 5′ primer (5′-CTCTGTAAC-CACTCACCAGC-3′) is located 96 bp upstream of the *Eco*RV site shown in *a*. The sequence of the 3′ primer (5′-CCTGC-GTGCAATCCATCTTG-3′) is located 320 bp downstream of the *Xho*I site in pMC1Neo-polyA. The lengths of diagnostic restriction fragments and hybridization probes are indicated. Probe A (*Nco*I–*Xho*I) and probe C (*Nco*I–*Nco*I) are derived from the $C\mu$ locus, probe B (*Xho*I–*Sal*I) from pMC1Neo-polyA. *B*, Southern blot analysis of D3 cells and PCR-positive transfectants (four clones—3, 4, 58 and 210—are shown). Genomic DNAs were digested by *Bam*HI and *Eco*RI. The filter was hybridized successively by the three probes indicated below each panel. *a*, In the PCR-positive clones, probe A hybridized to a wild-type 5.2-kb fragment as well as to 3.2- and 2.2-kb fragments in the targeted locus, as expected. *b*, Probe B hybridizing to 3.2- and 1.0-kb fragments shows the presence of the *neo*^{r} sequence in the locus. The faint 4.2-kb band is probably due to partial digestion of the DNA. *c*, As predicted, probe C, located outside the targeting vector, hybridized in the PCR-positive clones to the same 3.2-kb fragment as above as well as to the wild type fragments (6.6 and 5.2 kb). The *Cδ* locus remained intact in the mutated allele as shown by hybridization with a *Cδ*-specific probe (data not shown).

METHODS. D3 cells[13] (gift of E. Wagner) were grown on mitomycin C-treated G418-resistant STO fibroblasts[14] (gift of C. Kappen and F. Ruddle). The cells were electroporated with 20 $\mu g\ ml^{-1}$ of the linearized vector (*Ab*) as described[11]. The cells were plated at a density of 6×10^6 cells per 10-cm feeder plate and selected with G418 (200 $\mu g\ ml^{-1}$) and GANC (2 μM)[12]. As a control, 2×10^6 transfected cells were selected in G418 only, and gave rise to 110 G418^r colonies. After 11–14 days, G418^r–GANC^r colonies were screened for homologous recombination by PCR analysis as follows: the colonies were picked and trypsinized in multi-well plates individually. Half of the cells from each colony were cultured on multi-well plates. The other cells (8–12 colonies) were pooled for PCR analysis. Pooled samples were treated with proteinase K (ref. 23) and gene amplification was done in 50 μl 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.01% gelatine, 3 mM $MgCl_2$, 0.1 μM primer (*Ac*), 0.4 mM dNTPs and 2.5 U *Taq* polymerase for 40 cycles (1.5 min at 95 °C, 1 min at 65 °C and 2 min at 72 °C). Samples containing targeted cells gave a 1-kb amplified fragment. The clones from PCR-positive pools were then analysed individually and positive clones were expanded and analysed by Southern blotting (*B*).

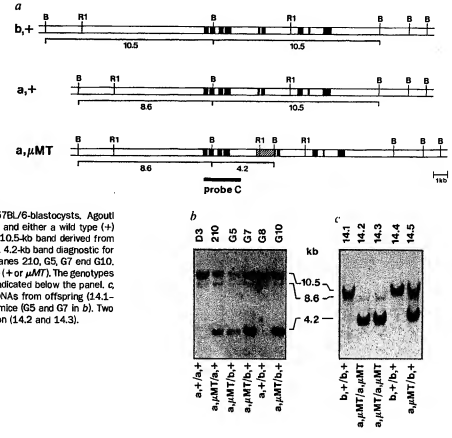

FIG. 2 *a* Genomic structure of the Cμ–Cδ loci of the *a* and *b* alleles and of the *μMT* mutant with predicted sizes of the fragments detected by probe C in Southern blot analysis (*b* and *c*). The Igh^b wild type allele (*b*,+; top) is from C57BL/6. The Igh^a wild type (*a*,+; middle) and mutated (*a*,*μMT*; bottom) alleles are from the mutated ES clones. *b*, Southern blot analysis showing germ-line transmission of the *μMT* mutation. DNAs from D3 cells (D3), a mutated D3-clone (2.10, Fig. 1*B*) and tails of agouti offspring (G5, G7, G8 and G10) were digested by *Bam*HI and hybridized with probe C. The offspring was from a C57BL/6 female mated to a chimaeric male which had been generated by the injection of clone 2.10 into C57BL/6-blastocysts. Agouti offspring carry an Igh^b allele of C57BL/6 origin and either a wild type (+) or a mutated (*μMT*) Igh^a allele of D3 origin. A 10.5-kb band derived from Igh^b or wild type Igh^a was present in all lanes. A 4.2-kb band diagnostic for the mutated allele (Igh^a, *μMT*) was present in lanes 2.10, G5, G7 and G10. A weak 8.6-kb band is derived from the Igh^a allele (+ or *μMT*). The genotypes of the Cμ loci determined by the analysis are indicated below the panel. *c*, Southern blot analysis of *Bam*HI-digested tail-DNAs from offspring (14.1–14.5) of an intercross of heterozygous mutant mice (G5 and G7 in *b*). Two animals were homozygous for the *μMT* mutation (14.2 and 14.3).

on the surface nor B cells in the peritoneal cavity (data not shown). In addition, the homozygous mutant mice had no detectable IgM in the serum (<0.1 μg ml^{-1}), compared with ~600 μg IgM per ml in the controls (data not shown). In contrast, *μMT*/*μMT* mice generate substantial numbers of T cells, as demonstrated by staining with anti-Thy-1 (Fig. 4*a*) or anti-CD3 (Fig. 4*b*) antibodies.

Which stage of B cell development is affected by the *μMT* mutation? B cells are continuously generated from stem cells in the bone marrow. The latter cells first differentiate into pre-B cells, detectable by flow cytometry as CD45R(B220)^{dull}, surface IgM$^-$(sIgM$^-$) cells[17]. Through rearrangement of the L chain genes, the pre-B cells give rise to CD45R(B220)^{dull}, sIgM$^+$ B cells[1,17]. In the bone marrow of the homozygous mutant mice sIgM$^+$ cells were absent (Fig. 4*c*). By contrast, the frequency of cells of the pre-B cell phenotype was roughly the same as in the controls, although the level of CD45R(B220) expression in these cells seemed slightly lower. Pre-B cells go through an early phase of proliferation as large cells from which small, non-dividing pre-B cells originate[18–20]. Analysis of the CD45R(B220)^{dull} cells in the homozygous mutant mice by forward light scatter showed that they were mainly large, in contrast

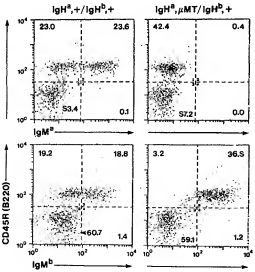

FIG. 3 Flow cytometric analysis (FACScan, Becton-Dickinson) of peripheral blood lymphocytes (PBL) from 3-month-old F_1 mice carrying (Igh^a, *μMT*/Igh^b, +; mouse G7 in Fig. 2*b*) or not carrying (Igh^a, +/Igh^b, +, mouse G8 in Fig. 2*b*) the *μMT* mutation. Peripheral blood lymphocytes were purified on a Ficoll-gradient and stained with phycoerythrin (PE)-conjugated monoclonal antibody RA3-6B2[24] (anti-CD45R(B220)) and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies, RS3.1[25] (anti-IgM^a) or MB86[26] (anti-IgM^b). Dots and percentages in the fluorescence windows refer to cells in the lymphocyte gate as defined by light scatter[17]. The vertical and horizontal axes show intensity of red (PE) and green (FITC) fluorescence, respectively.

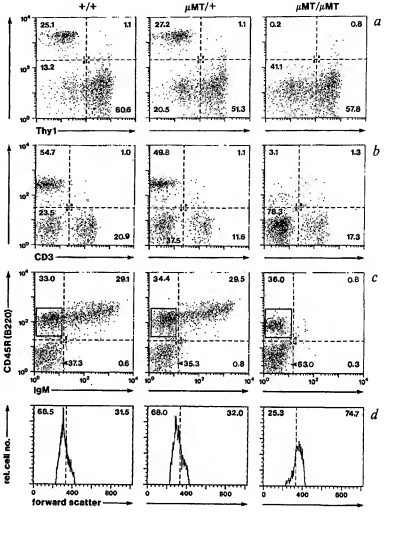

FIG. 4 Flow cytometric analysis of lymphocytes from homozygous mutant ($\mu MT/\mu MT$; 14.3 in Fig. 2c), normal (+/+; 14.4 in Fig. 2c) and heterozygous mutant ($\mu MT/+$; 14.5 in Fig. 2c) littermates. *a*, PBLs, *b*, spleen cells or *c*, bone marrow cells from 4-week-old mice were stained with monoclonal antibody RA3-3A1[19]-PE (anti-CD45R(B220)), and mAbs CFO 1[27]-FITC (anti-Thy 1, *a*) or 145-2C11[28]-FITC (anti-CD3, *b*) or R33-24-12[29] FITC (anti-IgM, *c*). Dead cells were excluded by propidium-iodide staining in *b* and *c*. (See also legend of Fig. 3.) The boxes in *c* define those cell populations reanalysed for cell size by forward light scatter as displayed in *d* in the form of histograms.

to the pre-B cells in controls, of which only a small proportion were large (Fig. 4*d*). Therefore, the μMT mutation seems to arrest B-cell differentiation at the pre-B cell stage, presumably close to, or at the point of, transition from large to small pre-B cell. But, it cannot be excluded that the CD45R(B220)^{dull} cells in the bone marrow of the homozygous mutant mice do not represent B-lineage cells; in this case the μMT mutation would prevent pre-B cell generation altogether.

The present work established that B-cell development is dependent on the expression of the membrane form of the μ chain by the pre-B cell stage. This implies a function for this protein before L chain expression and supports the idea that μ_m together with 'surrogate' L chains forms a membrane receptor through which pre-B-cell differentiation is controlled. Furthermore, the μMT mouse mutant both allows testing of the hypothesis that allelic exclusion is mediated through μ_m expression and offers itself as a model of an immunodeficiency selectively affecting B-cell development, as occurs in humans[21]. □

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DOCKET NO.: CARP0015-101 **PATENT**
APPLICATION SERIAL NO. 10/692,918

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Frank Grosveld	**Confirmation No. 9062**
Serial No.: 10/692,918	**Art Unit No.: 1632**
Filing Date: October 24, 2003	**Examiner: Anoop K. Singh**
For: IMMUNOGLOBULIN 1	
Customer No.: 34132	

DECLARATION OF LOUIS M. WEINER, M.D.

1. I have been engaged by Erasmus University to provide my opinion on certain issues concerning the above-identified application. I am being compensated at a rate of $ 450 per hour to do so.
2. My *curriculum vitae* ("cv") is attached as Exhibit A.
3. I received a Bachelor of Arts, cum laude, honors in biology, from the University of Pennsylvania in 1973. I received my medical degree from the Mount Sinai School of Medicine, N.Y., N.Y. in 1977.
4. I am currently the Vice President, Translational Research and Chairman, Department of Medical Oncology at the Fox Chase Cancer Center ("Fox Chase"), Philadelphia, Pennsylvania. I have been with Fox Chase since 1984. I have been Chairman of the Department of Medical Oncology since 1994; I have been Vice President, Translational Research since 2002.
5. As Vice President of Translational Research, my duties includeconnecting laboratory science with clinical investigation to improve the diagnosis and therapy of cancer. I oversee the allocation of research funds to support novel technology development and to stimulate collaborations among bench researchers and clinical investigators.
6. As is evident from my CV, I have been involved in immunology-focused research throughout my 30-year career, and have published extensively on immunology-focused

topics. I am a manuscript reviewer on numerous peer-reviewed journals including, for example, *Journal of Immunotherapy*, *Nature Biotechnology*, and *Science*. I have been an invited speaker at conferences regarding the protein engineering of monoclonal antibodies.

7. I am also the inventor on several patent applications, including U.S. Patent Publication No. 20040071696 involving bispecific antibodies.

8. I have reviewed the above-identified application. The correspondence with the examiner and the recent response from the inventor Dr. Grosveld. I am familiar with the involved technology, namely single heavy chain antibodies. Single heavy chain antibodies are antibodies composed of heavy chains only; no light chains are present. Functional heavy chain only antibodies are naturally produced by the camelid family and were first described by Hamers-Castermans in 1993 (Nature 363,446-448) **(copy attached)**. Camelid VHH heavy chain only antibodies are unable to bind light chain due to the absence of the CH1 domain.

9. As single heavy chain antibodies are smaller in size than antibodies comprising full-length heavy and light chains, there has been much interest generated in their use for therapy, in particular the VHH antigen binding domain. Accordingly, there has been much interest in developing antibodies against specific antigens for administration to mammals other than camelids including, of course, humans. Camelid antibodies are likely to produce an immune response in man, so either camelid antibodies must be "humanized" after isolation from the camelid, or alternative routes devised to generate human or "camelised" human heavy chain only antibodies more suitable for human therapy.

10. The present application reports, and claims, methods for producing single heavy chain antibodies in transgenic non-human mammals using the mouse as a model system. A immunoglobulin locus heavy chain locus engineered to prevent CH1 expression is introduced into the germ-line using well established technology. Heavy chain only antibody is produced in a B-cell specific manner as expected in response to antigen challenge.

11. To produce the antibodies, a transgene comprising a heavy chain locus comprising V, D

and J segments and heavy chain constant regions devoid of CH1 is expressed in a non-human mammal. I understand that one requirement, as recited in the claims, is that the heavy chain locus comprises at least one V exon, at least one D exon, and at least one J exon, capable of recombining to form VDJ coding sequences. The ability of the V, D, and J exons to recombine is extremely important, as this is one of the mechanisms that facilitate diversity in naturally produced antibodies . Another requirement, as recited in the claims, is that the heavy chain locus be expressed in B cells such that VDJ rearrangement occurs in specialized antibody producing cells. Another requirement of the invention, as recited in the claims, is that the heavy chain locus, when expressed, does not express a functional CH1 domain as part of the constant region.

12. All claims of the above-identified application are currently under rejection by the U.S. Patent & Trademark Office ("the Office"). I have read the Final Rejection dated February 12, 2007. I have also read the Interview Summary dated June 1, 2007; the Request for Reconsideration ("Request") filed June 25, 2007; and the Advisory Action Before the Filing of an Appeal Brief ("Advisory Action") dated July 9, 2007.

13. In the Final Rejection, the Office rejected the claims under the following bases:

    1. for lack of enablement under 35 USC § 112, first paragraph;
    2. for being incomplete as omitting essential steps under 35 USC § 112, second paragraph;
    3. as anticipated by Ledbetter et al (WO 99/42077) under USC § 102(b); and
    4. for obviousness-type double patenting over the claims of copending Application No. 10/692,918.

    I have been asked to address bases 1 and 3 above.

14. The first basis for rejection is further broken done into four issues. I will address the first three issues. The first issue appears to be that the specification only provides prophetic reference to most of the method steps, and does not provide any sequence information for the vectors (see page 6 of the Final Rejection). The Office's view is that undue experimentation would be required to practice the invention. I respectfully disagree.

15. I have read Janssens et al., *PNAS,* USA, 103(41)15130-15135, October 10, 2006. It is clear from the successful experiments reported in Janssens et al., which were performed

using the methods provided in the above-identified application, that the above-identified application provides sufficient detail for one of skill in the art to practice the invention.

16. The Office acknowledges the disclosure in Janssens et al. in the Advisory Action, but is limiting its effect. The Office seems to be taking the position that Janssens et al. is *only* enabling for expression of the GΔ construct (IgG heavy chain) in a wild type background, and is *only* enabling for the production of antibody in response to antigen challenge in GΔ construct-containing mutant mouse. The Office states that the guidance provided by applicants invites others to try different existing transgenic knockout nonhuman animals incapable of producing antibodies that include light chains. (See the Advisory Action, page 4.) The Office provides no scientific reason to conclude that the MΔGΔ construct, which Janssens et al. reports was expressed in the mutant mouse, would not also be expressed in wild type mouse. The Office also provides no scientific reason to conclude that the production of antibody in response to antigen challenge can only occur in mutant mice that do not produce light chain-containing antibodies. Regarding the first conclusion, I note that Janssens et al. report that the mutant mice produced some IgG, IgA, and IgE, i.e., antibodies that include light chains (p. 15130, col. 2, first full paragraph). Regarding the second conclusion, camelids produce both single heavy chain only antibodies and antibodies that include light chains, so it is clearly not necessary that the mammals be mutants that do not produce light-chain containing antibodies. As Janssens et al. confirms, the key components necessary for practicing the invention are set forth in the specification; the gene sequences introduced comprise all the necessary features required for the productive expression of heavy chain-only antibody (devoid of light chain) derived from heavy chain-only loci in the B-cells of transgenic mammals. The additional statement and data provided by Dr. Grosveld confirms this view. The data show that the MΔGΔ locus is expressed in wt mice and that human IgM is produced.

17. While the presence of suppressed endogenous heavy and light chain genes may be advantageous when selecting for antigen specific heavy chain-only antibodies against a background of endogenous immunoglobulin gene activity, it is clear that heavy chain only transgenes compete **successfully** with the endogenous genes active in the same B-cells, and allelic exclusion determines which loci will ultimately be used to express

functional antibody in the activated B-cell. Further, Janssens et al have shown that a camelised human locus comprising camelid VHH segments plus Human D and J segments plus human constant regions are functional and that camelised human heavy chain only antibody (IgM and/or IgG) circulates in plasma in genetic backgrounds where the endogenous mouse immunoglobulin genes are active, and that the same camelised human heavy chain only locus functions in a background (μMT) where endogenous mouse immunoglobulin gene activity has been suppressed. Moreover when the camelid VHH segments are replaced with human V segments, then fully human heavy chain only antibodies are produced in a wild type mouse background where the mouse heavy and light chain loci remain functional (See paragraph 5 of Dr. Grosveld's declaration, which I understand is being submitted concurrently.) The genetic background thus has no bearing on the enablement of this invention.

18. The second issue is that the production of functional camelid heavy chain is unpredictable. The Office cites a recently published reference for support of its position -- DeGenst et al, *Dev. Comp. Immunol.*, 30(1-2):187-98 (2006). The Office states that the reference reports that the timing and actual mechanism of class switching from mu to the dedicated gamma isotype in camelids remains elusive (see page 7 of the Final Rejection). Class-switching, however, is not a requirement of the method of the present invention. As Janssens et al. reports, the GΔ construct, which contains no mu exons, was successfully expressed (p. 15132). The Office also states that it is "not apparent from the specification whether a method as recited in claims would result in fully functional scIgG molecule" (see page 7 of the Final Rejection). Janssens et al., however, shows functional expression of both mu and gamma single heavy chain isotypes (Janssens et al Figure 6; three for IgM, many more for IgG, and all of these bind antigen, pp. 15132 and 15134).

19. The present invention is about the derivation of heavy chain only antibody in the B-cells of transgenic mammals. The inventor has defined the gene sequences to be introduced and has demonstrated that, provided the CH1 region is not expressed in the transgene(s), functional antibody is produced of the class or classes determined by the effector regions present in the transgene. This has been exemplified through the expression of heavy chain only IgM and IgG subtypes, or IgG subtypes alone, in different transgenic lines. It

is also clear that this is independent of the presence or otherwise of a functional endogenous immunoglobulin locus.

20. The third issue is that the claims cover the use of any promoter, with any regulatory elements, in any non-human transgenic mammal. The Office alleges that, at the time of invention (which I understand to be at least April 24, 2001, the earliest priority date claimed), only the mouse was recognized by the art of record as a routinely manipulated animal, and that the art of record recognized the unpredictability of making transgenic animals other than mice (see page 8 of the Final Rejection). I do not agree that the art of record shows this. The Office is focusing upon levels of expression sufficient to show a particular phenotype. In the present invention, it is only important that heavy chain-only antibody expression occurs in B-cells. All that is required is sufficient gene expression to isolate the antibody mRNA or antibody. As reported in Janssens et al., antibodies can be isolated and subsequently characterised by hybridoma production or phage display libraries (p. 15133). Dr. Grosveld's declaration shows that human heavy chain-only antibody mRNA can be isolated and sequenced from the B-cells present in a few microlitres of blood, and this against a background of B-cells producing endogenous mouse antibody. Similarly, human heavy chain-only antibody can be identified circulating in plasma.

21. Moreover, the techniques used (i.e., PCR amplification of mRNA and DNA sequencing; antibody detection by Western blotting) were routine laboratory tools at the priority date. The invention results in the production of novel antibodies. Once identified and characterized, heavy chain only antibodies or fragments thereof can be amplified and manufactured using established technologies of no relevance to this invention.

22. The second basis for rejection that I was asked to consider was the rejection for anticipation over Ledbetter et al. In the Final Rejection, the Office states that Ledbetter et al. anticipates claims 1 and 2. I am aware that, in order for a reference to anticipate a claim, it must disclose each and every limitation of the claim, and must be enabling. Specifically, the Office states that Ledbetter et al. discloses a method to produce VHH monoclonal and polyclonal antibodies in a transgenic animal in response to antigen challenge, citing page 33, para. 2-3 and page 34, para. 2 of Ledbetter et al. The Office

further states that, since Ledbetter et al. contemplated producing monoclonal antibody by immunizing the transgenic mice, expression of the VHH heavy chain locus in B cells in response to antigen challenge is inherent. (See Final Rejection, page 15.) I have reviewed Ledbetter et al. and disagree with the Office's assessment.

23. The concept of transgenic animals expressing llama VHH is first raised on p. 32 of Ledbetter et al. and refers to "the VHH sequences isolated by the methods disclosed herein." These are VHH binding domains derived from camelid mRNA and so are a result of transcription from already rearranged llama genes (see p. 44-46 section 8.1.2). Each VHH binding domain will comprise a VHH gene segment and D gene segment and J gene segment fused together as a single transcription unit. On p. 33, line 22, of Ledbetter et al., it is proposed that transgenic mice expressing VH binding domains "maybe used for the production of VHH to any antigen by immunizing transgenic animals with an antigen". This, however, is technically impossible -- the VHH binding domains are transcribed from already rearranged llama genes.

24. As is clear from the above, V, D, J rearrangement, and subsequent affinity maturation, occurs in the camel. This follows the teaching of Hamers-Casterman et al., referenced above, who first described camelid heavy chain only antibodies, and whose work is the subject of many published US patents describing the generation and subsequent use of camelid antibodies. See, for example, U.S. Patent Nos. 6,838,254; 6,765,087; 6,015,695; 6,005,079; 5,874,541; 5,840,526; 5,800,988; and 5,759,808. Ledbetter et al. then isolates a rearranged camelid gene (See Fig. 4) and proposes to reintroduce this into a transgenic mouse for the purpose of raising further novel antibodies in response to further antigen challenge. Such a gene, however, lacks all the features necessary for antibody diversity (e.g., multiple D and J regions) and cannot rearrange as it has been rearranged already. (See attached figure depicting the differences between Ledbetter et al. and the present invention.) Moreover, a recent publication from an independent laboratory has shown that, while such a rearranged gene can be expressed productively as a transgene in a mouse background (B-cell activation occurs) the resulting antibody retains all the features of the antibody originating from the camel (see Zou et al., "Expression of a dromedary heavy chain-only antibody and B cell development in the mouse," *J Immunol.*

2005 Sep 15;175(6):3769-79 **(copy attached)**). Thus, Ledbetter et al. fails to describe or generate a transgenic animal capable of producing heavy chain only antibody in response to antigen challenge.

25. As I understand it, claims 1 and 2 of the above-identified application are claiming the production of a single heavy chain antibody having a camelid (VHH) variable region. The antibody is produced in response to challenge with a particular antigen and, thus, binds to that antigen. Janssens et al. reports that single heavy chain antibodies against *E.* coli hsp70, *Bordetella pertussis, Tetanus* toxoid, rtTA, and TNFα were obtained using the method of the invention (p. 15133, col. 2, and p. 15135, col. 1). In contrast, Ledbetter et al. does not disclose a method to produce VHH monoclonal and polyclonal antibodies in response to antigen challenge. Ledbetter generates camelid VHH antibodies in llama and then displays recovered VHH binding domains by phage display (see Figure 1, **attached**). Accordingly, Ledbetter et al. does not enable the production of antibody in response to antigen challenge from a heavy chain only locus in a transgenic animal and, thus, cannot anticipate claims 1 and 2.

26. I hereby declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

11/15/07 _____

Date Dr. Louis M. Weiner

CURRICULUM VITAE

Updated 08/07

Louis M. Weiner, M.D.

NAME: Louis Marc Weiner

DATE OF BIRTH: May 21, 1951 - Philadelphia, PA

EDUCATION:

1973	University of Pennsylvania, Phila., PA.	B.A. cum laude with Honors in Biology
1977	Mount Sinai School of Medicine, N.Y., N.Y.	M.D.

CLINICAL TRAINING:

1977-1978	Medical Center Hospital of Vermont University of Vermont, Burlington, VT	Intern, Dept. of Medicine
1978-1980	Medical Center Hospital of Vermont University of Vermont, Burlington, VT	Resident, Dept. of Medicine
1980-1981	Medical Center Hospital of Vermont University of Vermont, Burlington, VT	Chief Resident, Dept. of Medicine
1981-1982	New England Medical Center Hospital Tufts University School of Medicine Boston, MA	Clinical Fellow, Hematology/Oncology
1982-1984	New England Medical Center Hospital Tufts University School of Medicine Boston, MA	Research Fellow, Hematology/Oncology

FACULTY POSTS:

Current:	Vice President, Translational Research Chairman, Department of Medical Oncology G. Morris Dorrance, Jr. Endowed Chair in Medical Science Senior Member, Division of Medical Sciences Fox Chase Cancer Center
1980-1981	Attending Physician, Department of Medicine, Medical Center Hospital of Vermont, Burlington, Vermont
1980-1981	Instructor, Department of Medicine, University of Vermont College of Medicine, Burlington, Vermont
1984-1990	Associate Member, Department of Medical Oncology Fox Chase Cancer Center, Philadelphia, PA

1985-1994	Director, Medical Oncology Fellowship Program, Fox Chase Cancer Center
1987-1990	Assistant Professor, Temple University School of Medicine
1991-1995	Member, Department of Medical Oncology Fox Chase Cancer Center, Philadelphia, PA
1991-1995	Associate Professor, Temple University School of Medicine
1994-Pres	Chairman, Department of Medical Oncology Fox Chase Cancer Center, Philadelphia, PA
1995-Pres	Senior Member, Department of Medical Oncology Fox Chase Cancer Center, Philadelphia, PA
1995-Pres	Professor, Temple University School of Medicine
1996-Pres	Director, Developmental Therapeutics Program Fox Chase Cancer Center, Philadelphia, PA
1998-2005	Chief, Section of Medical Oncology Department of Medicine, Temple University Hospital
2000-Pres	Consulting Staff, Division of Medical Oncology Department of Medicine, Jeanes Hospital
2002-Pres	Vice President, Translational Research Fox Chase Cancer Center

BOARD CERTIFIED:

1978	National Board of Medical Examiners
1980	American Board of Internal Medicine
1985	Medical Oncology

LICENSURE: Pennsylvania - #MD 031312E

AWARDS:

1985	American Cancer Society Career Development Award
1986	National Cancer Institute Clinical Investigator Award
1997	Janssen Pharmaceutical Research Foundation: Research Award for Outstanding Contributions to the Field of Receptor Targeted Therapy
2001	American Cancer Society-Southeast Region Scientific Research Award
2002	American Association for Cancer Research Scientist <-> Survivor Program Award
2002	G. Morris Dorrance, Jr. Endowed Chair in Medical Science
2000-2006	Best Doctors in America
2002-2005	Who's Who in Medicine and Healthcare
2002; 2004-2007	Philadelphia Magazine's Top Docs
2007	Elliott Osserman Award for Distinguished Service in Support of Cancer Research; Israel Cancer Research Foundation (ICRF) Review Panel 2005-2007

EDITORIAL BOARDS:

1996-	Editorial Board: Journal of Immunotherapy
1996-2000	Editor-in-Chief: Presentations in Focus/Oncology Forum
1996-	Tumor Board: Oncology Times
1998-	Editorial Board: Journal of the Int'l Society of Tumour Targeting
1999-	Editorial Board: Clinical Cancer Research
1999-	Editorial Board: Investigational New Drugs
2000-2002	Editorial Board: Journal of Clinical Oncology
2001-	Editorial Advisory Panel: Expert Review of Anticancer Therapy
2000-	Associate Editor: Cancer Research
2001-2002	Editor-in-Chief: PeerView in Focus
2001-2006	Editorial Board: Molecular Cancer Therapeutics
2001-	Editorial Board: Human Antibodies
2002-	Editorial Board: International Journal of Oncology
2003-	Editorial Board: In Vivo
2003-	Editorial Board: Advances in Targeted Cancer Therapies
2005-	Editorial Advisory Board: Medscape Hematology-Oncology Medicine

PEER-REVIEWED RESEARCH SUPPORT:

1986 National Cancer Institute - Clinical Investigator Award KO8 CA01130. - Principal Investigator 1986-1989

1989 National Cancer Institute - RO1-CA50633. "Antibody-Targeted Immunotherapy of Cancer." - Principal Investigator: 1989-2004

1990 National Cancer Institute-U01-CA51880 National Cooperative Drug Discovery Group. "Engineered Antibreast Cancer Single-Chain Fv Immunotoxin." - Program Leader 1990-1995

1992 Division of Cancer Treatment, NCI - Master Agreement.
RFP No. NCI-CM-27732-49, "Clinical Evaluation of Biological Response Modifiers (BRMs) for the Treatment of Cancer". - Principal Investigator.
NO1-CM-27732-01, "Phase I Evaluation of the Murine Bispecific Monoclonal Antibody 2B1" 1992 - 1993

1993 National Cancer Institute - U01 CA58262 - "Clinical Development of 2B1 Bispecific Monoclonal Antibody" Principal Investigator: 1993- 1997

1993 National Cancer Institute - K12 CA01728 - "Clinical Oncology Research Career Development Program" Principal Investigator: 1997-2003

1994 National Cancer Institute - U10 CA27525 - "Eastern Cooperative Oncology Group" Principal Investigator, Fox Chase Cancer Center: 1994-2004

1996 National Cancer Institute - R01 CA65559 - "Tumor Targeting by Single-Chain Fv Molecules" Principal Investigator: 1996-2003

1998 ARMY IDEA – DAMD17-98-1-8084 - "Fcγ Receptor-targeted Immunization for Breast Cancer" Principal Investigator 1998-2001

1998 ARMY Clinical Translational – DAMD17-98-1-8307 - "Targeting Breast Cancer with Anti-HER2/neu Diabodies" Principal Investigator 1998-2003

1999 ARMY – DAMD17-99-1-9183 – "Antibody-Pretargeted Cytokine Therapy of Cancer" Principal Investigator 1999-2003

2002 National Cancer Institute – R21 CA097461 – "Tumor and Endothelial Cells as Drug Targets in Blood" Principal Investigator 2002-2005

2003 National Cancer Institute – R41 CA099410 – "Gene Expression in Circulating Tumor Cells" Principal Investigator 2003-2004

2003 National Cancer Institute - RO1-CA50633-18. "Antibody-Targeted NK Cell Activation for Cancer." Principal Investigator: 1989-2008

2003 National Cancer Institute – K08 CA090468 – "Therapeutic Inhibition of Firbrblast Activation Protein 2003-2008

2004 National Cancer Institute – P50 CA083638 – "SPORE in Ovarian Cancer" - Co-Principal Investigator 2004-2009

2005 National Cancer Institute – P30 CA006927 – "Comprehensive Cancer Center Program at Fox Chase" Senior Leader and Major Program Leader 2005-2010

2005 National Cancer Institute – R42 CA099410 – "Gene Expression in Circulating Tumor Cells" Co-Principal Investigator 2005-2007

2006 National Cancer Institute – S10 RR019008 – "Guava EasyCyte Base System (Guava Personal Cell Analysis-96 System) 2006-2007

2006 National Cancer Institute – R01 CA107088 – "Bispecific Antibody Pretargeting for Therapy" 2006-2010

2006 National Cancer Institute – R21 CA121541 – "Analysis of Circulating Tumor Cells in a Phase I/II Study for Breast Cancer" 2006-2008

2006 American Cancer Society – MRSG-06-003-01-CCE "Circulating Tumor Cells to Develop Novel Pancreatic Cancer Therapy" 2006-2010

2006 National Cancer Institute – R01 CA121033-01 – "Adaptive Immunity from High Affinity Anti-HER2/neu Monoclonal Antibodies" Principal Investigator – 2006-2011

OTHER RESEARCH SUPPORT:

1985 American Cancer Society Career Development Award - Principal Investigator

1985 Biogen Corporation - "A Phase III Study Comparing Therapy Using Recombinant Interferon-gamma with Depoprovera in Advanced Renal Cell Carcinoma." - Co-principal Investigator

1986 Frank Strick Foundation - "Biological Therapy Support Facility Development" - Principal Investigator

1986 Mary L. Smith Charitable Lead Trust - "Development of Laboratory Models for Evaluating the Clinical Potential of Biologic Response Modifier Combinations" - Co-principal Investigator

1986 Benjamin Franklin Partnership - "Preclinical Evaluation of Magnetite-Antibody Congeners for Immunodiagnosis and Immunotherapy of Human Neoplasms" - Co-principal Investigator

1987 Frank Strick Foundation - "Development of Laboratory Models for Studying Ex Vivo Activation of Human Effector cells to Potentiate Monoclonal Antibody Therapy" - Principal Investigator

1987 Cetus Corporation - "Phase I Trial of 260F9-MAb-rRA Immunotoxin" - Principal Investigator

1991 Frank Strick Foundation - Clinical Development of Bispecific Monoclonal Antibodies - Principal Investigator

1991 Genentech - "Phase I Trial of M-CSF and γ-Interferon" - Principal Investigator

1991 Genetics Institute - "Phase I Trial of M-CSF and γ-Interferon" - Principal Investigator

1992 Frank Strick Foundation - "Clinical Development of Bispecific Monoclonal Antibodies" - Principal Investigator

1992 American Cancer Society - Clinical Oncology Fellowship - Program Director

1993 Chiron Corporation - "Phase I Trials of 2B1 Bispecific Monoclonal Antibody" - Principal Investigator

1993 Chiron Corporation - "Anti-tumor Activity of Antigen Fork Bispecific Antibodies" - Principal Investigator

1993 Frank Strick Foundation - "Clinical Development of Bispecific Monoclonal Antibodies" - Principal Investigator

1994 Chiron Corporation - "Phase I Trials of 2B1 Bispecific Monoclonal Antibody" - Principal Investigator

1994 Pharmacia Corporation - "Phase I Trial of the Immunotoxin LS4565" - Principal Investigator

1995 Frank Strick Foundation - "Clinical Development of Novel Bispecific Monoclonal Antibodies" - Principal Investigator

1995 Pharmacia and Upjohn - "Phase I Trial of Repeated Doses of the Immunotoxin PNU214565" - Principal Investigator

1995 Bristol-Myers Squibb - "Phase I Trial of BR96-Doxorubicin" - Principal Investigator

1996 Frank Strick Foundation - "Immunotherapy of Cancer using Antibodies and their Engineered Derivatives" - Principal Investigator

1997 Pharmacia and Upjohn - "Adaptive dosing based on circulating anti-SEA antibodies using PNU214565" - Principal Investigator

1997 Frank Strick Foundation-"Development and Clinical Testing of Novel Immunotherapeutics". - Principal Investigator

1997 Smith-Kline Beecham – "Topotecan Clinical Trials" – Principal Investigator

1998 Janssen Pharmaceutica – "Pre-Targeted Radioimmunotherapy" – Principal Investigator

1998 Janssen Pharmaceutica – "Phase I trial of the Farnesyl transferase inhibitor R115777" – Principal Investigator

1998-2006 Frank Strick Foundation - "Immunotherapy of Cancer using Antibodies and their Engineered Derivatives" - Principal Investigator

1999 Abgenix Inc. – "An Open Label, Maintenance Dosing, Clinical Trial of ABX-EGF in Patients with Renal, Prostate, Pancreatic, Non-Small-Cell Lung, or Esophageal Cancer" – Principal Investigator

2001 Genentech – "Clinical Training Award" – Principal Investigator

2005 Eisai Corporation – "TLR4 Agonist Promotion of Antibody-Promoted Adaptive Immunity" – Principal Investigator

2006-08 Amgen Corporation – "High-Throughput siRNA Screening" – Co-Principal Investigator

MANUSCRIPT REVIEWER:

Cancer
Cancer Research
Clinical Cancer Research
Institute of Medicine Report – External Reviewer: "Shortening the Time Line for New Cancer Treatments"
Investigational New Drugs
Journal of Cellular Pharmacology
Journal of Clinical Investigation
Journal of Clinical Oncology
Journal of Immunotherapy
Lancet
Nature Biotechnology
New England Journal of Medicine
Oncology: International Journal of Cancer Research and Treatment
Science

GRANT REVIEWER:

Division of Research Grants, National Cancer Institute

1. Special Review Committees, March 1990, August 1990, April 1991, July 1991
2. ad hoc Reviewer, Small Business Innovation Research, July 1990
3. Member, Experimental Therapeutics-2 Study Section, October, 1991-June, 1995.
4. ad hoc Reviewer, Study Section/Special Emphasis Panel to review RFA AI-00-006 "Innovative Grants on Immune Tolerance"
5. Member, NIH Clinical Trials Subcommittee C, 2001 - 2005.
6. ad hoc Reviewer, Study Section/Cancer Immunopathology and Immunotherapy-October, 2003.
7. Member, Cancer Immunopathology and Immunotherapy Study Section, December, 2004-June, 2008.

Grants Review Branch, Division of Extramural Activities, National Cancer Institute - NCI Center Support Grant Review Committee, June 1998

Dutch Cancer Society Review Board, The Netherlands, 1998-1999

Co-Director, American Cancer Society Institutional Review Committee, Fox Chase Cancer Center

External Advisory Committee, TUFTS/New England Medical Center's Training Grant in Clinical Care Research.

Cancer Panel Review Member, Doris Duke Distinguished Clinical Scientist Award (DCSA), 2002.

Chairman, UCSF U54 CA90788 External Advisory Committee, 2003 – Present

Member, Israeli Cancer Research Foundation (ICRF) Scientific Review Panel, 2004 - Present

Committee Member, Biologics Panel, NIH/NCI RAID, 2005 – Present

Committee Member, NCI/Translational Research Working Group (TRWG) 2005 - 2007

THESIS: Weiner, L.M.: Adaptive Responses of Mitochondria to Alterations in Ambient Oxygen Tension. Honors Thesis, Department of Biology, University of Pennsylvania; accepted May, 1973.

SELECTED INVITED LECTURES:

1986

Cancer and Science VII - Fox Chase Cancer Center - April, 1986

The Estelle Laska Memorial Series

The American Cancer Society - Chester County Unit - April, 1986

Sixth Annual Cancer Symposium - Western Reserve Care System - Youngstown, Ohio - April, 1986

Schreiner Symposium on Colorectal Cancer - Seattle, WA - October, 1986

Toward 2000, Philadelphia, PA, October, 1986

1987

Medical Grand Rounds - Temple University - February, 1987

Medical Grand Rounds - Robert Wood Johnson Medical School, Camden, New Jersey - October, 1987

1988

Biomodulation of Cancer - Society for Biological Therapy, San Francisco, California - November, 1988

1989

Review of Medical Oncology - Montefiore Medical Center, New York, New York - March 1989

Monoclonal Antibody Conjugates - UCSD Cancer Center, San Diego, California - March 1989

Tumor Conference - Christiana Medical Center, Wilmington, Delaware - October 1989

1990

Grand Rounds - Lankenau Hospital, Philadelphia, Pennsylvania - March 1990

13th Annual Cancer Update - Marquette General Hospital, Marquette, Michigan - March 1990.

Pittsburgh Cancer Institute Annual Symposium - University of Pittsburgh, Pittsburgh, PA - March 1990.

Bispecific Antibody Targeting Tumor and FcγRIII, 2nd International Conference on Bispecific Antibodies and Targeted Cellular Cytotoxicity, Seillac, France - October 1990.

1991

Biotherapy of Cancer: A Symposium for Clinicians and Nurses. Hoag Memorial Hospital, Newport Beach, CA. - February, 1991

European School of Oncology - Israel Cancer Association. Shoresh, Israel - October, 1991

1992

Eastern Cooperative Oncology Group Scientific Retreat - February, 1992

Toward 2000 VIII, Fox Chase Cancer Center, Philadelphia, PA - October, 1992

1993

FASEB Summer Conference, Saxtons River, VT - August, 1993

Monoclonal Antibody Therapy of Cancer, Contemporary Issues in Hematology/Oncology,

Univ. of Florida Health Science Center, Jacksonville, FL - November, 1993

Fourth Annual International Conference on Antibody Engineering, Coronado, CA - December, 1993

1994

28th Annual Main Line Conference, Bryn Mawr, PA, April 1994

Antibody-Based Therapeutics, Washington, D.C., June, 1994

Bispecific Monoclonal Antibody Therapy - Washington, DC - June 1994

1995

Session Chair, 4th International Conference on Bispecific Monoclonal Antibody Therapy, Hawks Cay, FL - March, 1995

Session Chair, Tenth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, CA - March, 1995

Chairperson, Poster Discussion Session, Bispecific Antibodies, Monoclonal Antibodies and Immunoconjugation, American Association for Cancer Research Annual Meeting, Toronto, Canada - March, 1995

Targeted Cancer Therapy Using Engineered Antibodies, Cancer Biology Seminar, Arizona Cancer Center, Tucson, AZ - October, 1995

Tumor-Targeting Properties of Anti-c-erbB-2 sFv, Therapeutic Antibody Technology 95 Symposium, San Francisco, CA - October, 1995

Session Chair and Speaker, Bispecific Anti-Her2/Neu Antibody Strategies, 10th Annual Meeting of the Society for Biological Therapy, Williamsburg, VA - November, 1995

Engineered Antibodies for Cancer Therapy, University of Pennsylvania Cancer Center, Philadelphia, PA - December, 1995

Targeted Therapy Using Engineered Antibodies, Multidisciplinary Clinical Conference, The Johns Hopkins Oncology Center, Baltimore, MD - December, 1995

1996

Session Chair and Speaker, Exploring and Exploiting Antibody and Ig Superfamily Combining Sites, Keystone Symposia, Taos, NM, February, 1996

Targeted Therapy of Cancer Using Engineered Antibodies, Robert L. Krigel Memorial Lectureship, Lankenau Hospital, Wynnewood, PA - April, 1996

Targeted Therapy Using Monoclonal Antibodies, University of Iowa Hematology-Oncology Grand Rounds, Iowa City, IA - May, 1996

Monoclonal Antibody Therapy of Human Malignancies, The Second Annual Northern New Jersey Cancer Center Research Symposium, Experimental & Clinical Approaches in Oncology: Approaching the 21st Century, Teaneck, NJ - May, 1996

Systemic Therapy for Rectal Cancer, Carcinoma of the Rectum: An Update for Physicians. William Beaumont Hospital, Royal Oak, MI - October, 1996

Session Chair and Speaker, Modulating binding affinity to improve the tumor-targeting properties of monomeric single-chain Fv molecules. IBC's Seventh Annual International Conference on Antibody Engineering, Coronado, CA - December, 1996

1997

Phase I trial of escalating doses of taxol in combination with cisplatin, fluorouracil and 60 Gy radiation prior to esophagectomy, Poster Discussion Session, The Fox Chase Cancer Center and Free University Hospital Investigator's Workshop and Consensus Conference, Rio Grande, PR - March, 1997

1997 Program Committee and Session Chair, Immunobiology and Gene Therapy Session, American Society of Clinical Oncology (ASCO), Denver, CO - May, 1997

Superantigen-targeted therapy: Phase I trials of PNU-214565, a fusion protein composed of staphylococcal enterotoxin A (SEA) and C242 Antibody Fab fragment in patients with advanced gastrointestinal malignancies, Speaker and Session Chairperson, IBC Conference on Antibody-Based Therapeutics, Boston, MA - June, 1997

Antibody-targeted activation of cellular immunity. Speaker at Symposium on The future use of monoclonal antibodies in cancer therapy at ECCO 9 -The European Cancer Conference, Hamburg, Germany - September, 1997

Targeted cellular therapy using bifunctional proteins. Department of Biochemistry, University of Virginia, Charlottesville, VA - October, 1997

1998

Antibody-directed superantigen therapy. Speaker, 11th International Conference on Monoclonal Antibodies for Cancer, San Diego, CA – March, 1998

Future directions: Review and outlook of Paclitaxel in esophageal cancer. Speaker, The Fox Chase Cancer Center and Free University Hospital Investigator's Workshop and Consensus Conference, St. Thomas, VI - March, 1998

Engineering antibodies for cancer therapy. Speaker, Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, Thira, Santorini, Greece – May, 1998

Engineered antibodies for cancer therapy. Speaker, NMHCC's Bio/Technology Division's Cancer Immunotherapy & Gene Therapy Conference, Arlington, VA – June, 1998

The development of monoclonal antibodies in medical oncology. Speaker, MD Anderson Cancer Center Medical Oncologist Consensus Conference on Future Directions with Herceptin for Breast Cancer, Wailea, Maui, HA – July, 1998

Faculty, ASCO/AACR Workshop on Methods in Clinical Cancer Research, Vail, CO – July, 1998

Breast cancer therapies. 13th annual Meeting of Society for Biological Therapy, University of Pittsburgh, Pittsburgh, PA – October, 1998

Biological therapy of cancer. 23rd Annual Michael Wohl Memorial Lecture, Temple University School of Medicine, Philadelphia, PA – November, 1998

Cancer Therapy using Engineered Antibodies. Speaker and Chairperson, IBC Ninth Annual International Conference on Antibody Engineering, Coronado, CA – December, 1998

1999

Clinical Strategies with Monoclonal Antibody Therapy, Speaker, Robert H. Lurie Comprehensive Cancer Center Oncology Consensus Conference on Recent Advances and Future Directions using Monoclonal Antibodies for B-Cell Malignancies, Kona, HA – January, 1999

Antibody therapy. Visiting Professor and lecturer, Course: "Viruses, Cancer, and Immunology", Dept. of Biological Sciences, Lehigh University, Bethlehem, PA – February, 1999

Manipulation of antibody/effector cell interactions. Participant, Monoclonal Antibodies in Oncology meeting, Genzyme Transgenics Corp., Framingham, MA – February, 1999

Monoclonal antibody therapy of cancer. 11th Annual Cancer Progress Conference, New York, NY – March, 1999

Therapeutic monoclonal antibodies for malignant disease. American Federation for Medical Research Symposium, Washington, DC – April, 1999

Novel Approaches in Pancreas Cancer Treatment. Marc Lustgarten Foundation for Pancreas Cancer Research, New York, NY – April, 1999

Targeted Cellular Cytotoxicity: The VIth Int'l Conference in Bispecific Antibodies and Related Strategies for Targeted Immune Modulation, Conference Co-Chair and speaker, Pacific Grove, CA – July, 1999

Faculty, ASCO/AACR Workshop on Methods in Clinical Cancer Research, Vail, CO – August, 1999

Bispecific Antibodies in Cancer Therapy. 1999 Oncology Frontiers Conference, Advisor and speaker, St. Thomas, US Virgin Islands - October, 1999

2000

Antibody-based fusion proteins as platforms for cancer therapy. Gordon Research Conference on Drug Carriers in Medicine & Biology, Ventura, CA - February, 2000

Immunotherapy with Cytokines and Naked Antibodies. International Conference on Advances in Cancer Immunotherapy: Organizing Committee, Session Chair and Speaker, Princeton, NJ - March, 2000

The Biological Therapy of Cancer. Grand Rounds, Graduate Hospital Department of Medicine, Philadelphia, PA – March, 2000

Predictors of Therapeutic Response/Discussion Session. Co-Chairperson Poster Discussion Session. American Association for Cancer Research annual meeting, San Francisco, CA – April, 2000.

Fellow Series: How to Write a Grant. Special Session presentation at annual American Society of Clinical Oncology, New Orleans, LA – May, 2000.

Treatment of epithelial tumors with monoclonal antibodies. Monoclonal Antibodies for the Treatment of Malignant Diseases: Present Achievements and Future Prospects. Speaker; Stockholm, Sweden – April, 2000

Biological therapy of cancer. Kitty Cookson Memorial Lecture. Royal Free and University College, London, England – July, 2000

Faculty, ASCO/AACR Workshop on Methods in Clinical Cancer Research, Vail, CO – August, 2000

Developmental Therapeutics. Keynote Speaker, Beth Israel-Deaconess Medical Center Annual Cancer Center Retreat, Woods Hole, MA – September, 2000

Treatment of Breast Cancer and Other Tumors with Monoclonal Antibodies. 59th Japanese Cancer Association, Tokyo, Japan – October, 2000

Cancer Therapy Using Engineered Antibodies. DuPont Pharma Seminar Series. Wilmington, DE – October, 2000.

Clinical and PreClinical Advances with Engineered Antibodies. Session Chair, IBC 11th Int'l Conference on Antibody Engineering, La Jolla, CA – December, 2000

Translation of Fundamental Advances in Immunology to Clinical Practice. Speaker and Chairperson, Scientific and Technological Innovations in Biology: Initiating Advances in Therapeutic Approaches to Hematologic Malignancies, American Society of Hematology, San Francisco, CA – December, 2000

2001

Novel Approaches to the Immunotherapy of Colorectal Cancer and Other Solid Tumors. Keystone Symposium on Molecular Medicine of Colorectal Cancer, Taos, NM – February, 2001.

Faculty, National Medical Oncology Fellows Forum. Orlando, FL – March, 2001.

Current Status of Antibodies in Cancer Therapy. 54th Annual Society of Surgical Oncology Symposium, Washington, DC – March, 2001.

Engineered Antibodies for Cancer Therapy. US-Japan Workshop on Recent Advances in Specific Immunotherapy of Cancer, Maui, HA – March, 2001.

Manipulation of the tumour microenvironment to facilitate antibody therapy. Speaker and Session Chairperson, VIIth International Antibody Conference on Targeted Cellular Cytotoxicity, Hampshire, UK – August, 2001.

Protein Engineering to Optimize Affinity and Efficacy of Monoclonal Antibodies. Congress on Monoclonal Antibodies, Banff, Canada – September, 2001.

ABX-EGF Receptor Antibody: Current Status and Future Directions. Congress on Monoclonal Antibodies, Banff, Alberta, Canada – September, 2001.

Antibody-based therapy of cancer. Research Grand Rounds, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL – November, 2001.

Manipulation of the tumor micro-environment to facilitate antibody therapy. IBC 12th Int'l Conference on Antibody Engineering, San Diego, CA – December, 2001.

2002

Monoclonal Antibodies. 43rd Annual Clinical Conference – Drug Discovery and Clinical Evaluation in the 21st Century, Houston, TX – January, 2002.

Antibody therapy of cancer – New concepts and approaches. Clinical and Translational Seminar Series, Cardinal Bernardin Cancer Center, Chicago, IL – February, 2002.

Tumor targeting by engineered antibodies. Interdisciplinary seminar series of the Institute for Medicine and Engineering (IME) of the University of Pennsylvania, Philadelphia, PA – March, 2002.

Discussant, Biologic and Targeted Therapies Oral Presentation Session. ASCO, Orlando, FL – May, 2002.

New antibody structures as therapeutic delivery vehicles. Planning Committee, Session Moderator and Speaker, 2nd Int'l Congress on Monoclonal Antibodies in Cancer, Banff, Alberta, Canada – August, 2002.

Protein engineering to optimize affinity of monoclonal antibodies. First Annual Symposium on Anti-Receptor Signaling in Human Neoplasia, Chicago, IL – September, 2002.

Engineered antibodies for cancer therapy. Excellence in Immunology Lecture Series, University of Texas Southwestern Medical Center at Dallas, Dallas, TX – December, 2002.

2003

Keystone Symposium: Antibody-Based Therapeutics for Cancer. Program Chairperson and Speaker, Banff, Alberta, Canada – February, 2003.

New approaches in monoclonal antibodies for cancer. XIV Cancer Progress Conference, New York, NY – March, 2003.

EGFR Antibodies: Are they different from the TK inhibitors? Speaker and Program Committee, 4th International Lung Cancer Congress, Maui, HA – June, 2003.

Faculty, ASCO/AACR Methods in Clinical Research Workshop, Vail, CO – July, 2003.

Immunology Program Chair and Speaker, 94th Annual Meeting of the American Association for Cancer Research, Washington, DC – July, 2003.

ABX-EGF: A high-affinity, fully human antibody to EGFR. Speaker, Session Moderator and Planning Committee Member, Third Int'l Congress on Monoclonal Antibodies in Cancer, Quebec, Canada – August, 2003.

Engineered antibodies for cancer therapy. Speaker, Int'l Society for Oncodevelopmental Biology and Medicine (ISOBM) 2003 Symposium, Edinburgh, UK – September, 2003.

Antibody therapy for the treatment of cancer. Speaker, 7th Int'l Meeting and 1st World Congress, Biotherapy of Cancer: From Disease to Targeted Treatment, Munich, Germany – September, 2003.

Monoclonal antibody therapy of cancer: Targeted therapy hits the target. Vermont Cancer Center Grand Rounds, University of Vermont, Burlington, VT – September, 2003.

Monoclonal antibodies in cancer therapy. Speaker, Cancer Education Consortium/ Genentech/IDEC Clinical Pharmacology of Anticancer Agents, Leesburg, VA – October, 2003.

Monoclonal antibody therapy - What's Next? Visiting Professor, EMD Pharmaceuticals, Inc., Durham, NC – October, 2003.

Approaches to the future treatment of solid tumors and metastatic disease in cancer. Speaker, Session Chair, and Scientific Advisory Board for IBC's 14th Int'l Antibody Engineering Conference, San Diego, CA – December, 2003.

Monoclonal antibody therapy: New targets for magic bullets. Division of Cancer Medicine Grand Rounds speaker, M.D. Anderson Cancer Center, Houston, TX – December, 2003.

2004

Program Co-Chair, Immunology Program Chair, Symposium Co-Chair and Sunrise Session Speaker, 95th Annual Meeting of the American Association for Cancer Research, Orlando, FL – March, 2004.

Monoclonal antibody therapy-What' Next? Session Leader, Master of Excellence in Medicine-Oncology Option, Institut Superieur de Formation A L'Excellence en Medecine, Videoconference to Paris, France – March, 2004.

Developmental Therapeutics: Immunotherapy Poster Session Discussant, 2004 Annual Meeting of American Society of Clinical Oncology, New Orleans, LA – June, 2004.

The Contributions of Nuclear Medicine in the Practice of Medical Oncology: Expectations of Medical Oncology. Society of Nuclear Medicine 51st Annual Meeting, Philadelphia, PA – June, 2004.

Faculty, ASCO/AACR Methods in Clinical Research Workshop, Vail, CO – August, 2004.

Evolving treatment options in colorectal cancer. Update on GI Neoplasia Conference, Philadelphia, PA – August, 2004.

Panitumumab (ABX-EGF): A high affinity, fully human antibody to EGFR. Speaker and Session Moderator, 4th International Congress on Monoclonal Antibodies Workshop, Colorado Springs, CO – September, 2004.

Preclinical and clinical development of recombinant antibodies. Session Chair and Scientific Advisory Board for IBC's 15th Int'l Antibody Engineering Conference, San Diego, CA – December, 2004.

2005

Antibody-based therapeutics for cancer: Keystone Symposia Program Co-Chair and Speaker, Santa Fe, NM – February, 2005.

The next generation in monoclonals - Who's on first? Sixteenth Annual Cancer Progress Conference, New York, NY – March, 2005.

Immunology and Immunotherapy: Special Interest Session. Scientist - Survivor Program, American Association for Cancer Research Annual Meeting, Anaheim, CA – April, 2005.

Chairperson, Targeted Strategies in Cancer Therapy: Improving Patient Outcomes, 2005 ASCO Satellite Symposium, Orlando, FL – May, 2005.

Presenter, Molecular Therapeutics Poster Session, and Discussant, Developmental Therapeutics: Immunotherapy Poster Session, 2005 Annual Meeting of American Society of Clinical Oncology, Orlando, FL – May, 2005.

Co-Chair Symposia Session #34, Antibodies: Innate versus Engineered, Era of Hope 2005/DOD Breast Cancer Research Program, Philadelphia, PA – June, 2005.

Faculty, ASCO/AACR Methods in Clinical Research Workshop, Vail, CO – August, 2005.

Panitumumab in Colorectal Cancer. Speaker and Session Moderator and Program Planner, 5th International Congress on Monoclonal Antibodies in Cancer, Quebec City, Canada – August, 2005.

New Directions in Cancer Treatment. Carcinoid/NET 2005 Conference, Philadelphia, PA – September, 2005.

Antibodies as the "Drug" for Cancer Therapy. Keynote speaker, Monoclonal Antibodies in Cancer, Philadelphia, PA – September, 2005.

Manipulating Antibody Affinity for Cancer Therapy: Too Much of a Good Thing? Speaker, Yale Cancer Center Grand Rounds, New Haven, CT – September, 2005.

Engineered Antibodies for Cancer Therapy. Speaker, Lankenau Hospital Fall Seminar Series, Wynnewood, PA – October, 2005.

Customizing Antibody Affinities for Cancer Therapy. Session Chair and Program Planner for IBC's 16th Int'l Antibody Engineering Conference, San Diego, CA – December, 2005.

2006

Current Clinical Approaches of Targeting EGFR in Colorectal Cancer. Faculty, Optimizing EGFR-Targeted Therapies in Colorectal Cancer Symposium, San Francisco, CA – January, 2006.

Engineered Antibodies for Cancer Therapy. Speaker, Carole and Ray Neag Comprehensive Seminar Series, University of Connecticut Health Center, Farmington, CT – February, 2006.

Customizing Antibody Affinities for Cancer Therapy. Sidney Kimmel Cancer Center Conference on Proteogenomics for Diagnosis, Imaging and Therapy of Cancer, San Diego, CA – February, 2006.

Immunology and Immunotherapy: Special Interest Session. Scientist - Survivor Program, 97th Annual American Association for Cancer Research, Washington, DC – April, 2006.

Customizing Antibodies for Cancer Therapy. Sunrise Session, 97th Annual Meeting of American Association for Cancer Research, Washington, DC – April, 2006.

Update on the Management of Colon Cancer. Lawrence M. Sigman, M.D. Memorial Lecture, Jeanes Hospital Department of Medicine; Philadelphia, PA - April, 2006

Frankly speaking about new discoveries in cancer: Special focus on colorectal cancer. 8th Annual Celebration of Hope; The Wellness Community of Philadelphia, Philadelphia, PA – June, 2006.

Faculty, FECS/AACR/ASCO Methods in Clinical Cancer Research, Flims, Switzerland – June, 2006.

Faculty, ASCO/AACR Methods in Clinical Research Workshop, Vail, CO – July, 2006.

Program Committee, Session Chair and Moderator and Speaker, 6th Int'l Congress on Monoclonal Antibodies in Cancer Symposium, Washington, DC – August, 2006.

Novel EGFR Inhibitors. Faculty, Current Trends in GI Malignancies Meeting, Philadelphia, PA – September, 2006.

Engineering antibodies for cancer therapy. Department of Immunology Grand Rounds, Roswell Park Cancer Institute, Buffalo, NY – September, 2006.

Engineering antibodies for cancer immunotherapy. UMDNJ/Cancer Institute of New Jersey Grand Rounds, New Brunswick, NJ – October, 2006.

Unconjugated antibodies for cancer therapy. Session Moderator and Speaker, 11th Conference on Cancer Therapy with Antibodies and Immunoconjugates, Parsippany, NJ – October, 2006.

Co-Chair, Mini-Symposium on Biologic Effects of Targeted Therapeutics and Session Co-Chair, 21st Annual Meeting of the Int'l Society for Biological Therapy of Cancer, Los Angeles, CA – October, 2006.

Monoclonal antibodies for cancer immunotherapy. Abramson Family Cancer Research Institute/Division of Hematology-Oncology at the University of Pennsylvania, Philadelphia, PA – November, 2006.

Session Chair, Immunoprevention. AACR Int'l Conference on Frontiers in Cancer Prevention Research, Boston, MA – November, 2006.

Conference Co-Chair, Tumor Immunology: An Integrated Perspective; AACR Special Conference in Cancer Research, Miami, FL – November, 2006.

Improving anti-tumor antibody-initiated ADCC. Faculty, IBC's 17th Annual Int'l Conference on Antibody Engineering: Antibody Engineering and Immunotherapeutics for the 21st Century, San Diego, CA – December, 2006.

2007

Cancer therapy using unconjugated monoclonal antibodies: Emerging concepts. LSU-Tulane Health Sciences Cancer Center Seminar Series, New Orleans, LA – January, 2007.

Engineered antibodies for cancer therapy. Drexel University School of Biomedical Engineering Seminar, Philadelphia, PA – February, 2007.

Promoting ADCC by natural killer cells. Keystone Symposium, Lake Louise, Alberta – February, 2007.

Obstacles to implementing cancer vaccines. NCI/FDA Workshop on Rapid Translation of Research Findings into Clinical Practice, Bethesda, MD – February, 2007.

Frankly speaking about new discoveries in cancer. The Wellness Community of Philadelphia, Philadelphia, PA – March, 2007.

New directions for monoclonal antibody therapy of cancer. Distinguished Scientists Seminar, Centocor Corp., Malvern, PA – March, 2007.

Engineered antibodies for cancer therapy. Sidney Kimmel Cancer Center of Johns Hopkins University, Baltimore, MD – March, 2007.

Session Chair, Educational Session "Toll Receptors and Cancer" at Annual Meeting of American Association of Cancer Research, Los Angeles, CA – April, 2007.

Engineering antibodies for cancer therapy. Keynote Speaker, The Future of Monoclonal Antibody Biotherapeutics Production and Development Symposium, New York Academy of Sciences, New York, NY – May, 2007.

MEMBERSHIPS/ADVISORY BOARDS:

National Committees

Eastern Cooperative Oncology Group (ECOG)
- Principal Investigator, Fox Chase Cancer Center (1996-2002)
- Chairman, Biologic Response Modifiers Committee (1996-2002)
- Member, Gastrointestinal Cancer Committee
- Member, Laboratory Science Committee
- Member, ECOG Board of Advisors
- Member, Audit Committee

Member, PDQ External Advisory Board - NCI
Member, PDQ Adult Treatment Editorial Board - NCI
American Association for the Advancement of Science (AAAS)
American Association for Cancer Research (AACR)
- Chairperson, Cancer Immunology Task Force
- Incoming Course Director, AACR/ASCO Clinical Methodology Workshop
- AACR Foundation Steering Committee
- Translational Research Committee
- Chair, Tumor Immunology Special Conference, November 2006
- Education Committee, 2007 Annual Meeting
- AACR Special Conferences Committee 2007-2010

American Society of Clinical Oncology (ASCO)
American Society of Hematology (ASH)
American Society of Immunology (ASI)
American Federation for Clinical Research (AFCR)
American Federation for Medical Research (AFMR)
Clinical Immunology Society (CIS)
Pennsylvania Oncologic Society (POS)
Society for Biological Therapy (SBT)
The Antibody Society (TabS)
External Advisory Board, Tufts-NEMC Cancer Center (2005 – present)
NCI RAID Program Review – September, 2005
NCI Translational Research Working Group (TRWG) (2005 – present)
- Parent Committee
- Prioritization Subcommittee

Other

Member, PROGRESS Editorial Advisory Board
Scientific Advisory Board, Immunotherapy Corporation (1998 – 2000)
Scientific Advisory Board, Cell Pathways Corporation (1998 – 2002)
Scientific Advisory Board, Millennium Pharm. (1999 – 2001)
Medical Advisory Board, Abgenix, Inc. (1998 – 2006)
Scientific Advisory Board, Celldex Therapeutics, Inc. (2005 – present)
Cancer Advisory Board, Serono Research Institute (2004 – present)
Hematology-Oncology Editorial Advisory Board, Medscape, LLC (2005-present)
Scientific Advisory Board, Merrimack Pharmaceuticals (2006 – present)
International Oncology Advisory Board, Johnson and Johnson (2006 – present)

ADMINISTRATIVE:

Member, FCCC Medical Science Division Appointments & Promotions Committee, 1992 - 1998.
Member, FCCC Intensive Care Unit Committee, 1986 - 1992.
Member, FCCC Laboratory Animal Committee, 1989 - 1994.
Director, FCCC Medical Oncology Fellowship Program, 1986 - 1994.
Director, FCCC Clinical Investigator Training Program, 1993 - Current.
Member, FCCC Executive Committee of Staff, 1994 - .
Member, FCCC Ambulatory Care Working Group Committee, 1994 - 2004
Co-Director, American Cancer Society Institutional Grant Review Board, 1995 - Present.
Member, FCCC Centerwide Appointments & Promotions Committee, 1999 - 2005
Member, Faculty Advisory Committee for Institutional Advancement, 2003 - Present.
Member, Internal Advisory Board of the Medical Outreach and Minority Affairs Program, 2005 - Present.
Member, Fox Chase/Temple Affiliation Executive Committee, 2005 – Present.
Member, FCCC Extramural Research Leadership Committee, 2006 – Present.
Member, FCCC Strategic Vision Committee 2007-
Member, FCCC Translational Research Committee 2007-

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PUBLISHED ARTICLES, REVIEWS, CHAPTERS:

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2. Nordheim A, Pardue ML, **Weiner LM**, Lowenhaupt K, Scholten P, Moller A, Rich A, Stollar BD. Analysis of Z-DNA in Fixed Polytene Chromosomes with Monoclonal Antibodies that Show Base Sequence-dependent Selectivity in Reactions with Supercoiled Plasmids and Polynucleotides. J Biological Chem.Vol 261 (1):468-476, 1986.

3. **Weiner LM**, Steplewski Z, Koprowski H, Sears HF, Litwin S, Comis RL. Biologic Effects of Gamma Interferon Pre-Treatment Followed by Monoclonal Antibody 17-1A Administration in Patients with Gastrointestinal Carcinoma. Hybridoma. 5 (Suppl. 1): 65-77, 1986.

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5. Schwob VS, **Weiner LM**, Hudes G, Ratech H. Extranodal Non-T cell Lymphoblastic Lymphoma in Adults: A New Clinicopathologic Entity. Human Pathology. Am J Clin Path. 90:602-605, 1988.

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9. **Weiner LM**. Biologic Response Modifiers in Cancer Treatment. Welcome Trends in Hospital Pharmacy. 10:7-12, 1988.

10. Gatenby RA, Moldofsky PJ, **Weiner LM**. Correlation of Tumor Oxygen Levels and Uptake of Radiolabeled $F(ab')_2$ Monoclonal Antibody Fragments in Metastatic Colon Carcinoma Radiology. 166:757-759, 1988.

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15. O'Dwyer PJ, Paul AR, Walczak J, **Weiner LM**, Litwin S, Comis RL. Phase II Study of Biochemical Modulation of 5-Fluorouracil by Low-Dose PALA in Patients with Colorectal Cancer. J Clin Onc. 8:1497-1503, 1990.

16. Garcia Palazzo IE, Gercel-Taylor C, Kitson J, **Weiner LM**. Potentiation of Tumor Lysis by a Bispecific Antibody That Binds to CA19-9 Antigen and the Fcγ Receptor Expressed by Human Large Granular Lymphocytes. Cancer Res. 50:7123-7128, 1990.

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Naturally occurring antibodies devoid of light chains

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RANDOM association of VL and VH repertoires contributes considerably to antibody diversity[1]. The diversity and the affinity are then increased by hypermutation in B cells located in germinal centres[2]. Except in the case of 'heavy chain' disease[3], naturally occurring heavy-chain antibodies have not been described, although antigen binding has been demonstrated for separated heavy chains[4] or cloned VH domains[5]. Here we investigate the presence of considerable amounts of IgG-like material of M_r 100K in the serum of the camel (*Camelus dromedarius*)[6]. These molecules are composed of heavy-chain dimers and are devoid of light chains, but nevertheless have an extensive antigen-binding repertoire, a finding that calls into question the role of light chains in the camel. Camel heavy-chain IgGs lack CH1, which in one IgG class might be structurally replaced by an extended hinge. Heavy-chain IgGs are a feature of all camelids. These findings open new perspectives in the engineering of antibodies.

By a combination of affinity chromatography on protein A and protein G, three quantitatively important fractions corresponding to subclasses of IgG can be isolated from the serum of camels (Fig. 1*A*, lanes c–f).

One fraction (IgG_1) contains molecules of M_r 170K (Fig. 1*B*, lane 2), which upon reduction yield 50K heavy chains and large 30K light chains (Fig. 1*C*, lane 2). The two other immunoglobulin fractions contain molecules of ~100K (Fig. 1*B*, lanes 1 and 3), which upon reduction yield only heavy chains of, respectively, 46K (IgG_2 fraction binding only to proin A) (Fig. 1*C*, lane 3) and 43K (IgG_3 fraction binding to protein A and protein G) (Fig. 1*C*, lane 1). These two IgG classes appear to lack the light chain completely.

To exclude the possibility that the light chains were only weakly associated with the heavy chains and lost during our selective purification, whole serum was size-fractionated by gel filtration. Coomassie blue staining of unreduced fractions revealed the sequential elution of the 170K IgG_1 followed by the incompletely resolved isotypes IgG_2 and IgG_3 (90K) (Fig. 1*D*, upper inset). Immunostaining of the same fractions after reduction confirmed that the light chains were present only in the 50K heavy-chain-containing fractions (Fig. 1*D*, lower inset).

A comparative study of old world camelids (*Camelus bactrianus and Camelus dromedarius*) and new world camelids (*Lama pacos, Lama glama and Lama vicugna*) showed that heavy-chain immunoglobulins are abundant in the sera of all species examined (data not shown) and total up to 75 per cent of the molecules binding to protein A.

The abundance of heavy-chain immunoglobulins in the serum of camelids raises the question as to whether they bear an extensive antigen-binding repertoire. This question could be answered by examining the IgG_1, IgG_2 and IgG_3 fractions from the serum of camels (*Camelus dromedarius*) with a high antitrypanosome titre[7]. In radio-immunoprecipitation, purified fractions of IgG_1, IgG_2 and IgG_3 derived from infected camels were shown to bind a large number of antigens present in a [^{35}S]methionine-labelled trypanosome lysate (Fig. 2*a*), indicating an extensive repertoire complexity for the three IgG classes. Conversely, in blotting experiments, [^{35}S]methionine-labelled trypanosome lysate binds to SDS-PAGE-separated IgG_1, IgG_2

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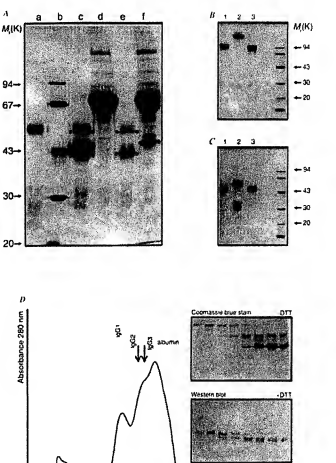


FIG. 1. Characterization and purification of camel IgG classes on protein A, protein G and gel filtration. *A*, The fraction of *C. dromedarius* serum adsorbed on protein A on reducing SDS–PAGE shows three heavy-chain components of 50, 46 and 43K (bands between dots), which are absent in the non-adsorbed fraction (lane d), and light-chain components of ~30K (lane c) that are considerably larger than rabbit light chain (lane a, rabbit IgG). The fractions adsorbed on protein G (lane e) lack the 46K heavy chain which remains in the non-adsorbed fraction (lane f). Lane b contains a size marker. *B* and *C*, By differential adsorption and elution on protein G and protein A, the IgG fractions containing 43K (lane 1), 46K (lane 3) and 50K (lanes 2) heavy chains were purified and analysed on SDS–PAGE in the absence (*B*) or presence (*C*) of dithiothreitol (DTT). *D*, Camel whole serum (0.1 ml) was fractionated by gel filtration on a Superdex-200 column using 150 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0, as eluent. Affinity-purified IgG_2 and IgG_3 elute at the positions indicated by arrows. Fractions of interest were analysed further by SDS–PAGE with or without prior reduction. The protein contents as visualized by Coomassie blue (without reduction; upper inset) are compared with the immunoglobulins from the same fractions (after reduction with DTT, lower inset) as revealed by western blotting with a rabbit anti-camel IgG (lower inset).
METHODS. *C. dromedarius* serum (5 ml) is adsorbed onto a 5-ml protein G–Sepharose (Pharmacia) column and washed with 20 mM phosphate buffer, pH 7.0. Upon elution with 0.15 M NaCl, 0.58% acetic acid (pH 3.5), IgG_3 of 100K is eluted, which upon reduction yields heavy chains of 43K (lanes 1 in *B* and *C*). IgG_2 of 170K can subsequently be eluted with pH 2.7 buffer (0.1 M glycine-HCl). This fraction upon reduction yields a 50K heavy-chain and a broad light-chain band (lane 2 in *C*). The fraction not adsorbed on protein G is run on a 5-ml protein A-Sepharose column. After washing and elution with 0.15 M NaCl, 0.58% acetic acid (pH 4.5), IgG_2 of 100K is obtained, which consists solely of 46K heavy chains (lane 3 in *C*).

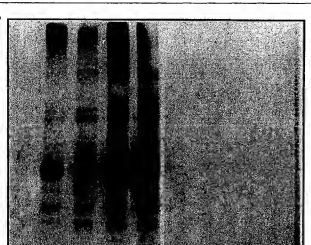
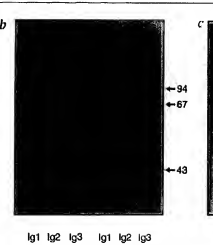
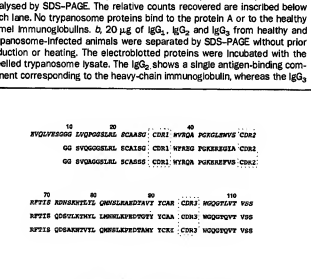


FIG. 2 Repertoire complexity and antigen-binding capacity of camel IgG_1, IgG_2 and IgG_3 analysed by *a*, radioimmunoprecipitation, or *b* and *c*, western blotting. *a*, Serum or purified IgG fractions from healthy or *Trypanosoma evansi*-infected *C. dromadarius* (CATT titre 1/160 (ref. 7)) were incubated with labelled trypanosome lysate, recovered with protein A–Sepharose and analysed by SDS–PAGE. The relative counts recovered are inscribed below each lane. No trypanosome proteins bind to the protein A or to the healthy camel immunoglobulins. *b*, 20 μg of IgG_1, IgG_2 and IgG_3 from healthy and trypanosome-infected animals were separated by SDS–PAGE without prior reduction or heating. The electroblotted proteins were incubated with the labelled trypanosome lysate. The IgG_2 shows a single antigen-binding component corresponding to the heavy-chain immunoglobulin, whereas the IgG_3 fraction appears in addition to contain two larger antigen-binding components barely detectable by Ponceau red staining (*c*). These could possibly be immunoglobulin classes copurified as immunocomplexes present in the serum of the infected animals.



METHODS. [^{35}S]methionine-labelled *Trypanosoma evansi* lysate (500,000 counts)[22] was incubated (4 °C, 1 h) with 10 μl serum or, 20 μg IgG_1, IgG_2 or IgG_3 in 200 μl of 0.4 M NaCl, 10 mM EDTA, 10 mM Tris, pH 8.3, containing 0.1 M *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK). Protein A–Sepharose (10 mg) suspended in 200 μl of the same buffer was added (4 °C, 1 h). After washing and centrifugation, each pellet was resuspended in 75 μl SDS–PAGE sample solution containing DTT and heated for 3 min at 100 °C. After centrifugation, 5 μl supernatant was saved for radioactivity counting and the remainder analysed by SDS–PAGE and fluorography. The nitrocellulose filter of the western blot of purified fractions IgG_1, IgG_2 and IgG_3 was stained with Ponceau red (*c*) or incubated with 1% ovalbumin in TST buffer (Tris 10 mM, NaCl 150 mM, Tween 0.05%) (*b*). The membrane was extensively washed with TST buffer and incubated for 2 h with ^{35}S-labelled trypanosome antigen. To avoid nonspecific binding, labelled trypanosome antigen lysate was filtered (45 μm) and incubated with healthy camel immunoglobulin and ovalbumin adsorbed on a nitrocellulose membrane.



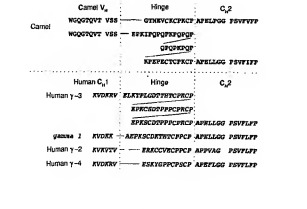
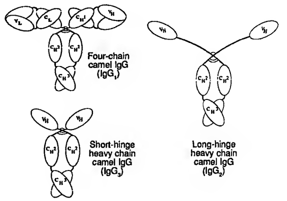


FIG. 3 Amino-acid sequences of the VH framework, and hinge/C_H2 of *Camelus dromedarius* heavy-chain immunoglobulins, compared to human (italic) VH framework (subgroup III) and hinges of human IgG[14].
METHODS. Total RNA was isolated from a dromedary spleen[23]. mRNA was purified with oligo(T) paramagnetic beads (PolyATract, Promega). 1 μg mRNA was used for preparing double-stranded cDNA[23] after an oligo(dT) priming using enzymes from Boehringer Mannheim. 5 ng cDNA was amplified by PCR in a 100-μl reaction mixture (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatine, 200 μM of each dNTP). 25 pmol of each oligonucleotide of mouse V_H[24], containing an *Xho*I site, and 5′-CGCCAT-CAAGGTACCAGTTGA-3′ were used as primers. The 3′-end primer was deduced from partial sequences corresponding to γ-chain amino acids 296 to 288 (T. Atarhouch, C. H.-C. and G.R., unpublished results) in which one mismatch was introduced to create a *Kpn*I restriction site. After a round of denaturing annealing (94°C for 5 min and 54°C for 5 min), 2 U of *Taq* DNA polymerase were added to the reaction mixture before 35 cycles of amplification[25]. PCR products were purified by phenol–chloroform extraction followed by HPLC (Genpak-fax column, Waters) and finaly by MERMAID (BIO101). After purification, amplified cDNA was digested with *Xho*I and *Kpn*I, and ligated into pBluescript. Clones were sequenced by dideoxy chain termination[25]. Sequences were then translated so that they could be assigned to well defined domains of the immunoglobulin molecule[14].

FIG. 4 Schematic representation of the structural organization of the camel immunoglobulins (adapted from ref. 26). On the basis of size, the IgG_1 fraction probably has the normal antibody assembly of two light and two heavy chains. IgG_3 would have a hinge comparable in size to the human IgG_1, IgG_2 and IgG_4. The two antigen-binding sites are much closer to each other as camel IgG lacks the CH1 domain. In camel IgG_2 the long hinge, which is formed of Pro-X repeats (X=Glu, Gln or Lys), probably adopts a rigid structure[19,20]. This long hinge could therefore substitute for the CH1 domain and bring the two antigen-binding sites of IgG_2 into normal positions.



and IgG_3 obtained from infected animals (Fig. 2*b*, *c*). These findings indicate that the heavy chains alone can generate an extensive repertoire and question the obligatory contribution of the light chain to the useful antibody repertoire in the camelids.

The camelid γ2 and γ3 chains are considerably shorter than the normal mammalian γ or camel γ1 chains. This would suggest that, as in the case of 'heavy chain' disease[3], deletions have occurred in the CH1 protein domain[8,9]. To address this question, complementary DNA was synthesized from camel spleen messenger RNA and the sequences between the 5' end of the VH and the CH2 were amplified by polymerase chain reaction (PCR), and cloned. Seventeen clones presenting a different VH sequence were isolated and sequenced. Their most striking feature was a complete lack of the CH1 domain, the last framework (FR4) residues of the VH region being immediately followed by the hinge (Fig. 3, lower part). The absence of the CH1 domain clarifies two important dilemmas. First, immunoglobulin heavy chains are normally not secreted unless the heavy-chain chaperoning protein or BIP (ref. 10) has been replaced by the L chain[11], or alternatively the CH1 domain has been deleted[3,8,9]. Secondly, isolated heavy chains from mammalian immunoglobulins tend to aggregate, but are only solubilized by light chains[8,12] which bind to the CH1 and the VH domains[13].

Fourteen of the seventeen clones were characterized by a short hinge sequence with a length equal to that of human IgG_2 and IgG_4 (ref. 14) (Fig. 3). The other three had a long hinge sequence containing the 'EPK' hinge motif found in human IgG_1 and IgG_3 (ref. 14). They possess the CH2 'APELL/P' motif that is also found in human IgG_1 and IgG_3, and which is associated with mammary transport of bovine IgG_1 (ref. 15). On the basis of their molecular weights, we expect the 'short-hinge' clones to correspond to IgG_3 and the 'long-hinge' clones to IgG_2.

In the short hinge-containing antibody, the extreme distance between the extremities of the VH regions will be of the order of 80 Å, corresponding to twice the size of a single domain of 40 Å (2×VH)[16]. This could be a severe limitation for agglutination, crosslinking or complement fixation[17,18]. In the long-hinge-containing immunoglobulin, the absence of CH1 might be compensated by the extremely long hinge itself, composed of a 12-fold repeat of the sequence Pro-X (where X is Gln, Glu or Lys) (Figs 3 and 4). NMR (ref. 19) and molecular modelling[20] of Pro-X repeats present in the TonB protein of *Escherichia coli* (in which X is Glu or Lys) and of the membrane procyclin of trypanosomes (X is Asp or Glu) indicate that these repeated sequences function as rigid rod-like spacers with a diameter of 8 Å and a rise of 2.9 Å per residue. Assuming the same geometry, the long hinge would be 70 Å, which compensates for the absence of the CH1 domain.

The binding site of heavy-chain antibodies cannot form the pocket resulting from adjoining light and heavy chain V regions, and the residues of VH that normally interact with VL will be exposed to solvent[3,5,13]. Leucine at position 45 is conserved in 98% of human and murine VH sequences[14] and is crucial in the VH-VL association[13]; it can be replaced by an arginine (Fig. 3, top section). This substitution is in accordance with both the lost contact with a VL domain and an increased solubility.

Unlike myeloma heavy chains, which result mainly from CH1 deletion in a single antibody-producing cell[21], the camelid heavy-chain antibodies have emerged in a normal immunological environment and will probably have undergone the selective refinement in specificity and affinity that accompanies B-cell maturation[1,2]. The obtention of camelid heavy-chain antibodies could therefore be an invaluable asset in the development and engineering of soluble VH domains[5] or of new immunological molecules for diagnostic, therapeutic and biochemical purposes. □

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Expression of a Dromedary Heavy Chain-Only Antibody and B Cell Development in the Mouse[1]

Xiangang Zou,* Jennifer A. Smith,* Viet Khong Nguyen,[2†] Liming Ren,[3*] Kattie Luyten,* Serge Muyldermans,[†] and Marianne Brüggemann[4*]

In mature B cells of mice and most mammals, cellular release of single H chain Abs without L chains is prevented by H chain association with Ig-specific chaperons in the endoplasmic reticulum. In precursor B cells, however, surface expression of μ-H chain in the absence of surrogate and conventional L chain has been identified. Despite this, Ag-specific single H chain Ig repertoires, using μ-, γ-, ϵ-, or α-H chains found in conventional Abs, are not produced. Moreover, removal of *H* chain or, separately, *L* chain (κ/λ) locus core sequences by gene targeting has prevented B cell development. In contrast, H chain-only Abs are produced abundantly in Camelidae as H2 IgG without the C_H1 domain. To test whether H chain Abs can be produced in mice, and to investigate how their expression affects B cell development, we introduced a rearranged dromedary γ2a H chain into the mouse germline. The dromedary transgene was expressed as a naturally occurring Ag-specific disulphide-linked homodimer, which showed that B cell development can be instigated by expression of single H chains without L chains. Lymphocyte development and B cell proliferation was accomplished despite the absence of L chain from the BCR complex. Endogenous Ig could not be detected, although V(D)J recombination and IgH/L transcription was unaltered. Furthermore, crossing the dromedary H chain mice with mice devoid of all *C* genes demonstrated without a doubt that a H chain-only Ab can facilitate B cell development independent of endogenous Ig expression, such as μ- or δ-H chain, at early developmental stages. ***The Journal of Immunology*, 2005, 175: 3769–3779.**

In the conventional mouse and human immune system, B cell development is initiated by VDJ recombination and surface IgM expression (Ref. 1, and refs. therein). At the pre-B cell stage, the associated surrogate L chain is replaced with a κ- or λ-L chain, and this initiates the process of Ab maturation, which is accompanied by cellular migration and class switching. At this stage, mature B cells undergo further selection and affinity maturation and can differentiate into Ab-secreting plasma cells or memory cells bearing other isotypes (IgG, IgA, or IgE). Developmental progression is blocked at the pre-B-I cell stage when H chain expression is prevented, although H and/or L chain transcripts may be found (2, 3). Likewise, silencing of both κ- and λ-*L* chain loci blocks B cell development, but at the somewhat later pre-B-II stage, which allows normal development up to pre-BCR expression (4). With the lack of L chain, μ-H chain is retained in the cytoplasm of immature bone marrow B cells, and their further development, with subsequent migration and colonization of the spleen, is prevented (4). Targeted modification of the *IgH* locus has permitted expression of truncated Ig polypeptides (5), and introduction of Ig transgenes consisting of shorter chains or removed domains has allowed single chain expression (6–8). Recently, it has also been shown that entire μ-H chains in association with the Igα coreceptor, but lacking surrogate or conventional L chain, can be expressed on the cell surface of pre-B cells. Single μ-H chain expression may induce differentiation signals and allow developmental progression possibly up to the immature B cell stage (9–11). Nevertheless, there are no examples where individual H chain polypeptides, on the surface or released from the cell without associated L chain, facilitate B cell differentiation to the mature and specialized stage leading to Ab repertoire formation in the mouse.

Abs, consisting of multiple units of paired H and L chains (12), emerged early in vertebrate evolution, and their presence is demonstrated in all of the jawed vertebrates studied to date (13). In addition to these conventional heteromeric Abs, sera of camelids (suborder Tylopoda, which includes camels, dromedaries, and llamas) contain a major type of Ig composed solely of paired H chains (14). Homodimeric H chain Abs in camelids lack the first C domain (C_H1) but harbor an intact variable domain (V_HH) encoded by different, clearly distinguishable, *V* genes (15). Using structural analysis, it has been concluded that it is impossible for a V_HH to pair with a normal V_L because the V_L-interacting side of the domain is reshaped by the hydrophilic V_HH hallmark amino acids and the long CDR3, which folds over this region (16). H chain Abs are absent in other mammals except in pathological cases, known as heavy chain disease, where parts of the V_H domain and/or C_H1 exon have been removed (17). Interestingly, H chain Abs are also present in some primitive fish, e.g., the new Ag receptor in the nurse shark and the specialized H chain (COS5) in ratfish (18, 19). Evolutionary analysis showed that their genes emerged and evolved independently, whereas *H* chain genes in camelids evolved from pre-existing genes used for conventional heteromeric Abs (20).

In camelids, the problem of developmental progression when single H chains are expressed may be circumvented because of

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structural differences in particular *V* and *γ-C* genes. As in conventional Ab production, the *H* chain gene of a H chain Ab is obtained after DNA rearrangements when specific $V_H H$ germline genes, located within the V_H gene cluster, are assembled with commonly used *D* and J_H segments to code for the V_HH-domain (21). Genomic and cDNA analyses have revealed five functional dromedary γ genes, three of which (γ*2a*, γ*2c*, and γ*3*) are always used to form H chain-only isotypes, whereas two separate genes, γ*1a* and γ*1b*, are used for the production of heterodimeric IgG isotypes (22). Only a very low yield of H chain-only Ab transcripts (identified by their particular *V* genes) spliced to Cμ ($V_H HDJ_H$-Cμ) have been identified from dromedary spleen (23). Serum IgM devoid of L chains has not been found, and staining of camelid B cells for IgG H chain-only Ab is not yet possible due to a lack of specific Abs. These observations indicate that the IgM stage of H chain Abs may be transient and that the conventional differentiation events initiated by IgM expression may be circumvented.

The lack of C_H1 in H chain Abs is most likely to be the crucial factor in allowing their release from cells in the absence of L chains. Although H chain *C* region genes encode the first exon, it is spliced out during mRNA maturation, probably due to a point mutation at the canonical splicing donor site (24, 25). It has been established that the C_H1 domain participates actively in the regulation of assembly and secretion of conventional H2L2 Abs. The nascent-translated H chain polypeptide associates noncovalently with the H chain binding protein (BiP[5] or grp78) via BiP association sites in C_H1 (26). The BiP/H chain complex is retained in the endoplasmic reticulum by virtue of the KDEL sequence at the carboxy terminus of BiP (27), and an H chain with a C_H1 domain is not secreted unless BiP is displaced by the L chain (26, 28, 29).

It is possible that H chain-only Abs have been selected and maintained in Tylopoda for their complementary function in recognizing unusual epitopes, such as clefts on the Ag surface that are normally less antigenic for conventional Abs (30). So it could be argued that H chain Abs are maintained because they fulfill a complementary function in their humoral immune response. In the peripheral blood of all camelids, H chain Abs of different isotypes contribute to the immune response; they undergo Ag-mediated selection and affinity maturation, and their $V_H HDJ_H$ domains are subjected to extensive somatic hypermutation (21, 22).

Our experiments focus on two essential questions. Can a dromedary H chain gene be processed and correctly expressed in the mouse? A feasibility study to evaluate the prospect of generating therapeutic camel H chain Abs in appropriately engineered mice is crucial to allow the future creation of H chain Ab repertoires. And, are conventional developmental processes capable of permitting B cell maturation without L chain association? This is important because L chain-dependent development or coexpression may impede the desired production of high-affinity dimeric H chains with their prospective use as enzyme inhibitors.

In this article, we show that H chain-only Ab expression of a rearranged dromedary γ*2a* gene in transgenic mice initiates B cell development without involvement of L chain or production of endogenous IgM. Allelic feedback signals are operative and seem to secure isotype exclusion by preventing expression but not V(D)J rearrangement and transcription of endogenous H and L chain genes. Multimeric IgG2a H chains are efficiently secreted, and their assembly on the cell surface allows Ag recognition. Expression of H chain Abs, with their particular configuration of the $V_H H$ gene, their C_H1 exon removed by splicing, and lack of L chain, does not appear to be regulated by the presence of a classical BCR complex.

Materials and Methods

Derivation of mice

The dromedary $V_H H$-*Cγ2aTM* H chain Ab gene on an 11.7-kb *NotI-SalI* fragment (31) was purified using a DNA purification kit (no. 28304; Qiagen). For the derivation of transgenic mice, DNA was microinjected into the male pronucleus of F_1 embryos (CBA × C57BL/6) according to standard methods (32), and several founders were produced. Transgenic animals were identified by PCR using dromedary Cγ2a-specific oligonucleotides: C_HH1 forward, 5′-GCACCTCGACCGGAAAGACCTTCATCTCC-3′; and hinge reverse, 5′-GGGACACGTCATTCTGGTTCAGG-3′, which produced a 485-bp fragment (data not shown). Two animals, judged by PCR and Southern hybridization to be high (cam1) and low (cam2) copy number founders, were bred to homozygosity with μ*MT* mice (33) and Cλ mice, in which all *C* genes had been removed by Cre-*loxP*-mediated deletion (3). Southern blot analysis (34) was conducted on *KpnI* digests of tail DNA hybridized with a 1.4-kb *NotI-BstEII* $V_H H$-gene probe (31).

Nucleic acid preparations and PCR

For transcriptional analysis of transgene expression, mRNA was prepared from bone marrow and spleen cells using the RNeasy Mini Kit (no. 74104; Qiagen), and cDNA was prepared with the Omniscript RT Kit (no. 205111; Qiagen) according to manufacturer's instructions. RT-PCR primers for identifying transgenic expression (see Fig. 2) were as follows: V3FR1B (V_HH), 5′-GAGGTGCAGCTGGTGGCGTCTGGAGGAGG-3; G2AH1F (H, hinge), 5′-GGGACACGTGCATTCTGGTTCA-3′; H (hinge) forward, 5′-CAACCAAAACCTGAACCAGAATGC-3′; C_HH2 reverse, 5′-GCACCTCAACGCCATCAATG-3′; C_HH3 forward, 5′-CAAGGACACCG TGAGCATAACCT-3′; 3′C_HH3 reverse (downstream of the stop codon), 5′-TGCCGGGGTGAGGCTCATTTA-3′; and M2 reverse, 5′-GCC CGATCATGTTTCTGTAGTCTG-3′. Lamin B1 served as a control to normalize the DNA concentration and also allowed discrimination of cDNA and genomic PCR products (3). PCR conditions were 93°C for 2 min followed by 32 cycles of 30 s at 93°C, 45 s at 58°C, and 90 s at 72°C, followed by 10 min at 72°C to complete the reaction.

For the analysis of D-J_H, V_H-D-J_H, and V_κ-J_κ-C_κ rearrangement and transcription, as well as surrogate L chain transcription, RNA was prepared from bone marrow and spleen cells using Tri Reagent (Sigma-Aldrich), and cDNA was prepared using the Omniscript RT kit as above. Genomic DNA was prepared by lysis with proteinase K at 55°C, phenol/chloroform/isoamyl alcohol (25/24/1; Sigma-Aldrich) extraction and ethanol precipitation. Combinations of the following oligonucleotides were used: for the H chain, a 1/1 mixture of DF (5′- GCATGTCTCAAAGCACAATG-3′) and DQ52 (5′-ACCCTGGA CACAGGAAACAC-3′); VJ558L (5′-ATGGGATGGAGCTGGATCTT-3′) for DNA; and a 1/1 mixture of VJ558L and VJ558CL (5′-ATGGAATG GAGCTGGGTCTT-3′) for cDNA and VJ183 (5′-ATGAACTTCGGGCT CAGCTT-3′) forward primers in combination with J_H1-4 reverse primer (5′-GAGACDGTGASHRDRGTBCCTKSRCC-3′) (3); for the L chain V_κ forward (5′-GGCTGCAGSTTCAGTGGCAGTGGRTCWGGRAC-3′) (35); universal Jκ (5′-GTTTKATTTCCARYYTKGTSCC-3′) and Cκ reverse (5′-GCTCATGCTGTAGGTGCTGTCTTTGCTGTC-3′); and for surrogate L chain Λ5 forward (5′-AGTTCTCCTCCTGCTGCTGCTGT-3′); Λ5 reverse (5′-TACCTTCCAGTCCACCACCAAAG-3′); VpreB forward (5′-TGGT CAGGKCCCAGGAGCAGTGG −3′); and VpreB reverse (5′-CCGGAGC CCCACRGCRCAGTAAT-3′). PCR conditions were 30–35 cycles of 93°C for 20 s, 62–64°C for 30–35 s, and 72°C for 40–50 s, followed by 10 min at 72°C to complete the reaction, and RT-PCR conditions were 2 min at 94°C followed by 32 or 33 cycles of 93°C for 20 s, 62–64°C (70°C for Λ5 and Vκ-Cκ, 50°C for J558-J_H1-4 RT-PCR) for 30–45 s, and 72°C for 50–60 s, followed by 10 min at 72°C to complete the reaction. The hybridization probe for the V558-J_H PCR was a 450-bp *EcoR*I fragment from a cloned J558 family member, kindly supplied by A. Wood (Babraham Institute).

ELISA and Western blot analyses

Serum Abs were identified by ELISA (36) on Falcon plates (no. 353911; BD Biosciences) coated with 10 μg/ml goat anti-llama IgG (no. A160-100A; Bethyl Laboratories). Bound Abs were detected either with HRP-conjugated goat anti-llama IgG (no. A160-100P; Bethyl Laboratories) or biotin (BIO)-conjugated rat anti-mouse κ L chain (no. 04-6640; Zymed Laboratories) or BIO-conjugated anti-mouse $\lambda_{1,2,3}$ L chain (BD Pharmingen) developed with streptavidin-biotinylated HRP (no. RPN1051; Amersham Biosciences).

[5] Abbreviations used in this paper: BiP, H chain binding protein; BIO, biotin; HEL, hen egg lysozyme; KO, knockout.

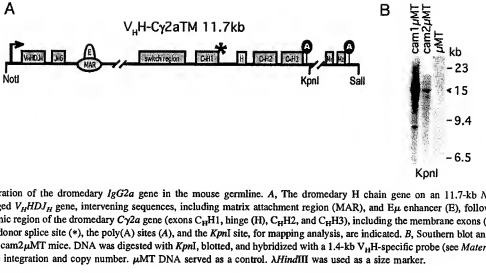

FIGURE 1. Integration of the dromedary *IgG2a* gene in the mouse germline. *A*, The dromedary H chain gene on an 11.7-kb *NotI-SalI* fragment comprises a rearranged V_HHDJ_H gene, intervening sequences, including matrix attachment region (MAR), and Eμ enhancer (E), followed by the switch region and the genomic region of the dromedary *Cγ2a* gene (exons C_HH1, hinge (H), C_HH2, and C_HH3), including the membrane exons (M1 and M2) (31). The mutation in the donor splice site (•), the poly(A) sites (*A*), and the *KpnI* site, for mapping analysis, are indicated. *B*, Southern blot analysis of tail DNA from cam1 μMT and cam2 μMT mice. DNA was digested with *KpnI*, blotted, and hybridized with a 1.4-kb V_HH-specific probe (see *Materials and Methods*) to identify transgene integration and copy number. μMT DNA served as a control. λ*HindIII* was used as a size marker.

Goat anti-llama IgG (1 mg/ml) was coupled to CNBr-activated Sepharose 4B (no. 17-0430-01; Amersham Biosciences) in 0.1 M sodium carbonate buffer (pH 8.5) and stirred gently overnight at 4°C. Coupled Sepharose was left in 1 M glycine for 2 h and then washed and stored in PBS 0.5% sodium azide. Serum (20 μl) was incubated with ~20 μl of anti-llama IgG Sepharose overnight, and unbound proteins were removed by washing with PBS. For Western blot analysis, proteins were separated on precast 4–15% Tris-HCl Ready-Gels (no. 161-1104; Bio-Rad) and transferred to nitrocellulose membranes as described previously (31). Filters were incubated with HRP-conjugated goat anti-llama IgG, or BIO-conjugated rat anti-mouse κ L chain or anti-mouse $\lambda_{1,2,3}$ L chain, followed by incubation with streptavidin-biotinylated HRP as described above, and chemiluminescent substrate (SuperSignal West Pico, no. 34080; Pierce) was used for detection according to the manufacturer's protocol. Restore Western blot stripping buffer was used in some experiments (no. 21059; Pierce). The m.w. marker was All Blue Standards (no. 161-0373; Bio-Rad).

Flow cytometry analyses

Bone marrow and spleen cell suspensions were prepared from cam1μMT, cam2μMT, μMT, cam1, cam2, cam2Cλ, RAG2$^{-/-}$, and normal F_1 mice. Cells were stained in combination with allophycocyanin-conjugated anti-mouse CD45R (B220) (no. 01129A; BD Pharmingen), FITC-conjugated anti-mouse IgM (no. 04-6811; Zymed Laboratories), PE-conjugated anti-mouse *c-kit* (CD117) (no. 09995B; BD Pharmingen) and/or BIO-conjugated anti-mouse CD43 (no. 01602D; BD Pharmingen), FITC-conjugated anti-mouse IgM, PE-conjugated anti-mouse Igκ (no. 559940; BD Pharmingen), FITC-conjugated anti-mouse IgA (no. 021174D; BD Pharmingen), FITC-conjugated anti-mouse CD21/35 (no. 553818; BD Pharmingen), and BIO-conjugated hen egg lysozyme (HEL) (31). Reactions with BIO-conjugated Abs were subsequently incubated with Tri-color-conjugated streptavidin (no. SA1006; Caltag Laboratories). Cytoplasmic staining was conducted using a fix and perm cell permeabilization kit with reduced background formulation (GAS-004; Caltag Laboratories) according to the manufacturer's instructions. Cells were analyzed on a FACSCalibur (BD Biosciences), and CellQuest (BD Biosciences) was used for data analysis. A FACSAria (BD Biosciences) was used for sorting B220$^+$ and B220$^-$ lymphocyte populations at up to 12,000 cells per second, which resulted in a purity >95%.

Results

Integration of a dromedary H chain gene construct in the mouse germline

The H chain Ab gene, V_HH-*Cγ2aTM*, has been constructed using a rearranged dromedary V_HHDJ_H gene with specificity for HEL and a dromedary *Cγ2a* gene in germline configuration, including the transmembrane exons (31). Figure 1*A* shows the 11.7-kb *NotI-SalI* fragment, which was microinjected into fertilized mouse oocytes. From the animals born, two were selected (cam1 and cam2, identified by PCR) for further breeding. These represented high copy number and low copy number transgenic founder mice. The animals were crossed with μMT mice in the C57BL/6 background (33), which resulted in cam$^{+/-}$ μMT$^{-/-}$ and μMT$^{-/-}$ animals, which were used for detailed analyses. In Southern blotting, *KpnI* digests showed several bands containing the transgene (Fig. 1*B*) with a predominant ~15-kb fragment, suggesting multiple and tandem integration as the construct harbors a ~7.4-kb *NotI-KpnI* fragment. Comparison of signal intensities estimated that the cam1μMT mice have the transgene integrated at a high copy number (>40 tandem copies), whereas cam2μMT is a low copy number (≥2 copies) line.

H chain transcripts are correctly spliced

An important question was whether the introduced H chain gene would be transcribed and whether the resulting product would match the transcripts found in Camelids. To investigate possible mRNA splice products, we used RT-PCR and sets of oligonucleotides that would reveal the exon usage (Fig. 2). Employment of V forward and hinge reverse oligonucleotides revealed a product of 500 bp from cam1 and cam2 mice (Fig. 2*A*), which corresponded to V_HHDJ_H-hinge splice products lacking the C_HH1. Inclusion of the C_HH1 exon would have increased the size to ~800 bp (31). The hinge region is followed by C_HH2 (Fig. 2*B*). Transcripts that allow Ig secretion (Fig. 2*C*) and surface expression (Fig. 2*E*, *top bands*) were found, both in bone marrow and spleen cells, as has been described for μ mRNA products in mouse B cells (37). Figure 2, *D* and *F*, illustrate the exon usage of the PCR products, which have been confirmed by cloning and sequencing (supplemental Table I).[6] Simultaneous amplification of lamin B1 (Fig. 2*E*, *bottom bands*) served as a semiquantitative reference and suggested that secretory and membrane transcript levels are higher in spleen and bone marrow cells from cam2 mice. In cam1 mice, we found little transmembrane product and a diminished intensity of the amplification bands. This implies low transcription levels of the dromedary transgene in cam1 mice, which may be due to the integration site, e.g., in a transcriptionally silent region, and is reaffirmed by ELISA and flow cytometry analysis shown below. Despite differences in expression levels, the results show correctly spliced H chain products, without C_HH1, in bone marrow and spleen, which

[6] The online version of this article contains supplemental material.

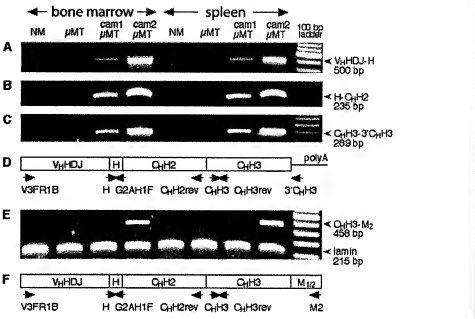

FIGURE 2. Transcription of dromedary H chain in bone marrow and spleen. RT-PCR analysis was conducted using oligonucleotides priming in the following regions: *A*, $V_{H}H$ and hinge (H); *B*, H and $C_{H}H2$; *C*, $C_{H}H3$ and 3′ of $C_{H}H3$ stop codon; *E*, $C_{H}H3$ and membrane exon 2 (M2) and lamin, as control conducted in parallel, to verify matching cDNA concentrations. *D* and *F*, Maps established from the product sizes. These show that the $C_{H}H1$ exon is omitted and that the correctly transcribed dromedary H chain consists of V_{H}-HDJ-H-$C_{H}H2$-$C_{H}H3$-M1/2. The secreted (*D*) and membrane (*F*) forms are found in both bone marrow and spleen cell populations. As a size marker, a 100-bp ladder was used. In addition, the exact size of each band was established by DNA sequencing (supplemental Table I).

implies that the introduced dromedary *H* chain gene is faithfully expressed in both secreted and transmembrane form.

Multimeric Ig is secreted in serum

To analyze secretion of dromedary H chain Ig, we captured serum Abs from the camμMT mice in a sandwich ELISA using goat anti-llama IgG for detection. Figure 3 illustrates strong Ab binding of two representative cam2μMT mice (termed a and b), the low copy transgenic line, with good detection of up to 1/1000 dilution. The high copy line, cam1μMT, had a low Ab titer (detectable only in 1/3 (data not shown) and 1/10 serum dilutions), whereas background binding was obtained when using μMT and normal mouse serum. Because binding to anti-llama IgG did not reveal the assembly of the secreted dromedary IgG2a, we further tested serum Abs for the presence of L chain. None of the cam1μMT, cam2μMT, and μMT sera showed binding to anti-mouse Igκ or anti-mouse Igλ L chain; however, normal mouse serum revealed some cross-reactivity, in that weak binding to anti-llama Ig could be detected with anti-Igκ.

To assess the assembly and m.w. of the secreted H chain Ig, we conducted Western blot analyses. To overcome a high background,

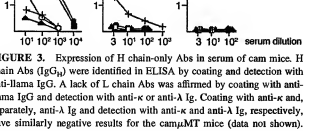

FIGURE 3. Expression of H chain-only Abs in serum of cam mice. H chain Abs (IgG_{H}) were identified in ELISA by coating and detection with anti-llama IgG. A lack of L chain Abs was affirmed by coating with anti-llama IgG and detection with anti-κ or anti-λ Ig. Coating with anti-κ and, separately, anti-λ Ig and detection with anti-κ and anti-λ Ig, respectively, gave similarly negative results for the camμMT mice (data not shown). The low copy number line cam2μMT showed good levels of H chain IgG with no L chain. This line is represented with serum titrations of two mice (a and b, ~3-mo-old) chosen from four separate experiments with similar results, using serum from at least eight mice, each between 6 wk and 10 mo of age. Normal mouse serum (NM) showed some cross-reactivity.

pronounced by the presence of serum albumin and separation under reducing conditions, we coupled anti-llama IgG to Sepharose for the purification of H chain Abs. Bound Abs from serum were separated on 4–15% polyacrylamide gels and visualized with HRP-coupled anti-llama IgG. The results (Fig. 4*A*) showed under reducing conditions a major band of ~46 kDa in cam1μMT and cam2μMT mice. A second, fainter, band of ~64 kDa is only seen in cam2μMT mice. Cloning and sequencing of a 1.6-kb fragment, as compared with the normal 1.2-kb band, obtained from RT-PCR using V3FR1B ($V_{H}H$) and 3′$C_{H}H3$ oligos, revealed an aberrant splice product due to tandem integration of the construct, incorporating an extra $V_{H}HDJ$ domain (supplemental Table I). This particular dromedary H chain is made up from $V_{H}HDJ$-$V_{H}HDJ$-H-$C_{H}H2$-$C_{H}H3$, which would add ~18 kDa to the normal size and explains the additional band obtained in Western analysis. However, this band is not a product using the $C_{H}1$ exon as verified by RT-PCR. The samples separated under reducing conditions showed faint H and L chain background bands from IgG coupled to Sepharose due to leakage (38). A reason could be that the different preparations of anti-llama Ig used for capture and visualization allowed some cross-detection, possibly enhanced by the sensitivity of the Western analysis. Separation of captured Ig under nonreducing conditions (Fig. 4*A*, *right*) revealed a major band of ~91 kDa, which represents H chain dimers. There are two larger bands, one of ~112 kDa and a much fainter band of ~135 kDa, which are likely to account for different multimers. Although the separation suggests that the secreted dromedary IgG2a H chain Ab produced in cam mice is largely associated as H2 homodimer, it may also associate as H3 multimer and, depending on resulting transcription products, in extended (2 × 64 kDa) or unequal (46 + 64 kDa) form. The longer exposure used to visualize H chain Ig products from cam1μMT mice is due to the lower levels produced (see Fig. 3).

Because no endogenous Ig could be identified in serum from camμMT mice, we conducted further Western separation on cam2 mice bred into the normal mouse background. Serum samples were applied to the gel in different amounts to allow a meaningful comparison. Figure 4*B* shows that no Igλ or Igκ could be detected in total cam2 serum, whereas significant amounts of dromedary H chain Ig remained as the only serum Ab. In summary, serum from

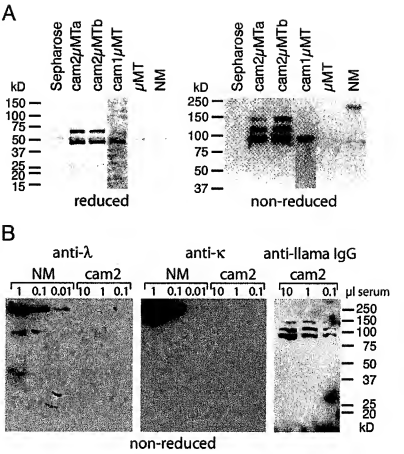

FIGURE 4. Western blot analysis of dromedary IgG2a. *A*, Serum Abs were captured by incubation with anti-llama IgG coupled to Sepharose, separated on Ready Gels, and stained with HRP-conjugated anti-llama IgG. Reducing conditions (*left*) revealed a ~46-kDa H chain band for cam2μMT (from mouse a and b) and cam1μMT (which was visible after longer exposure), and an additional band of ~64 kDa for cam2μMT. The fainter bands of ~26 kDa and ~53 kDa in all of the samples are the result of leakage of the Ab-coupled Sepharose (38) and cross-reactivity of different batches of anti-IgG, which contain IgG themselves. Samples separated under nonreducing conditions (*right*) revealed a major band of ~91 kDa (H2 configuration) for cam1μMT and cam2μMT. Additional bands found for cam2μMT of ~112 kDa and ~135 kDa may represent other multimers. Negative controls to affirm the specificity of the detection were anti-llama IgG-coupled Sepharose incubated with PBS, normal mouse (NM), and μMT serum. *B*, Total serum in 10-fold dilutions from cam2 in the normal mouse background and NM was separated under nonreducing conditions. Detection with anti-λ and anti-κ did not identify associated or residual L chain in cam2 mice, whereas development with anti-llama IgG showed the expected dromedary H chains. The filter was stripped between reactions. Detection with anti-κ necessitated a short exposure because of the strong NM signal. However, even longer exposures did not reveal any L chains in cam serum. The sizes of the marker bands are indicated.

camμMT or cam2 mice did not reveal any free or differently associated L chain by identification with anti-L chain reagents in ELISA and Western blotting, which showed that camelid H chain Ig can be exclusively produced in a mouse.

Progression of B cell development in bone marrow and spleen without L chain

A central question was how B cell development could progress in mice that express H chain Ig without L chain. Flow cytometry analysis of bone marrow cells (Fig. 5 left) from normal, μMT, and cam1μMT mice showed quite similar levels of pro- and pre-B cells, c-*kit*$^+$B220$^+$, and CD43$^+$B220$^+$, which are increased in cam2μMT mice (10% compared with 5% in normal mice or 4% in μMT mice). The reduced level of immature B220$^+$CD43$^-$ B cells in cam1μMT mice, 2% compared with 46% in a normal mouse, is barely detectable. However, cam2μMT mice, the low copy number but high expresser line, showed good recovery of the B lymphocyte population (14% compared with 46% B220$^+$ cells), which suggests induced B cell recovery by expression of the dromedary H chain gene. The effect is more dramatic in the spleen of cam2μMT mice (Fig. 5, *right*), where recovery of mature Ab-producing B cells reached about half the normal levels as shown in stainings with anti-CD21/35. Only poor recovery, 1.1%, is found in cam1μMT mice in concordance with the low level of expression found in this line. There is no expression of mouse IgM or Igκ or Igλ (data not shown) L chains. In the experiments, age-matched 3-mo-old mice were used. But very similar results were obtained, in flow cytometry, ELISA, Western, and PCR detection, when separate and parallel comparisons of 3-mo-, 6-wk-, and 6–10-mo-old animals were conducted. These results show that introduction of the dromedary H chain gene reconstitutes B cell development in the μMT or IgM-negative background without a requirement for L chain.

The H chain BCR signals exclusion of endogenous Ig

The purpose of crossing the dromedary H chain transgenic mice into the μMT background was to visualize H chain production without interference of mouse Ig. Initially, this was important, because the Abs that recognized the dromedary γ2a H chain cross-reacted with mouse Ig (see Figs. 3, and 4*A*). However, from the detailed analysis of the camμMT mice and serum Ig comparisons with cam2 mice bred into the normal mouse background, it became clear that dromedary H chain was well expressed and that B cell development progressed without L chain production. These results prompted further investigations to determine whether Ag-specific H chain Ig could be expressed on the cell surface and in the cytoplasm solely on its own or whether expression was accompanied by endogenous H or L chain polypeptides. Staining of bone marrow and spleen cells from cam2 transgenic mice with labeled HEL showed the presence of the Ag receptor on the cell surface of B220$^+$ cells (Fig. 6*A*). Binding of HEL was conducted in parallel using cells from MD-4 transgenic mice, which express HEL-specific Abs of high affinity (39). Although prominent surface staining with HEL was achieved in cam mice, the intensity was reduced compared with that of the MD-4 mice, perhaps due to low H chain density or reduced affinity. Receptor expression was not accompanied, even in the normal mouse background, by surface expression of endogenous IgM or IgL. Because this did not rule out the presence of endogenous Ig intracellularly, for example by chaperone retention, we used cytoplasmic staining. The results in Fig. 6*B*

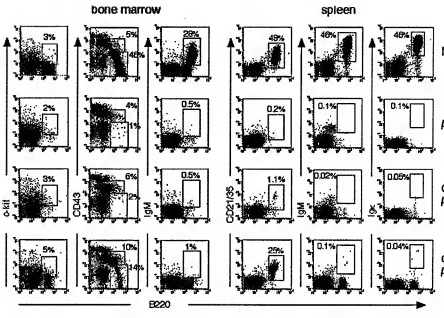

FIGURE 5. B cell development in mice expressing H chain-only Abs. Bone marrow (*left 3 rows*) and spleen cells (*right 3 rows*) from normal mice (NM), μMT, cam1μMT, and cam2μMT mice were stained with Abs against B cell differentiation markers. B220 served as a universal B cell marker and in combination with c-*kit*, and CD43 identified pro- and pre-B cells, which were well maintained in cam1μMT and cam2μMT mice. IgM is only expressed in normal mice, but cam2μMT mice showed good recovery of immature B220$^+$ cells, which are lacking in μMT and cam1μMT mice. Near normal levels of CD21/35$^+$ mature B cells were present in the spleen of cam2μMT mice (25%), but no L chain was found. The histograms were chosen from one of six independent experiments with very similar results, using ~3-mo-old mice.

showed HEL-specific Abs without the presence of mouse Ig. Unfortunately, some nonspecific background remains with this method (see *Materials and Methods*), which could point to residual mouse Ig expression at low levels. However, a similar background staining is found when using mice without endogenous C genes (3), reemphasizing that endogenous Ig levels are negligible.

H chain-only Abs are equally well expressed in C_H locus deletion mice

The findings that cam transgenic mice express dromedary H chain-only Ig in the absence of any mouse Ig chains and yet maintain appropriate levels of B220$^+$ cells, raised the question of whether endogenous H chain genes may be important for the early developmental stages. For example, pre-B cell development could be facilitated by VDJ recombination and endogenous H chain expression (μ, or δ in the μMT mice) initially accompanied but later replaced by transgene expression. It has been shown that the introduction of a rearranged murine γ transgene does not promote B cell development because joint expression with endogenous μ is required (40, 41). This finding suggested that IgG could not replace IgM, and it was speculated that their feedback signals to control B cell maturation must be different. To determine whether the dromedary γ2a H chain could be expressed without the help of other Ig genes, we crossed the cam2 mice with a recently derived line (CΔ$^{-/-}$) where all constant region genes had been deleted (3). These animals cannot express any H chain isotypes. As can be seen in Fig. 6, *A* and *B*, cam2CΔ$^{-/-}$ mice show the same level of B cells as cam2 mice and do not express any L chain. Indeed, it is worth noting that lymphocyte development, B cell levels, and Ab expression were very similar in camμMT$^{-/-}$, cam, and

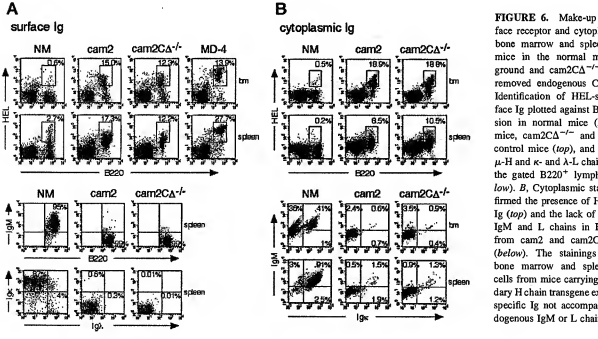

FIGURE 6. Make-up of the surface receptor and cytoplasmic Ig in bone marrow and spleen of cam2 mice in the normal mouse background and cam2CΔ$^{-/-}$ mice with removed endogenous C_H locus. *A*, Identification of HEL-specific surface Ig plotted against B220 expression in normal mice (NM), cam2 mice, cam2CΔ$^{-/-}$ and MD-4 (39) control mice (*top*), and stainings of μ-H and κ- and λ-L chain shown for the gated B220$^+$ lymphocytes (*below*). *B*, Cytoplasmic stainings confirmed the presence of HEL-specific Ig (*top*) and the lack of endogenous IgM and L chains in B220$^+$ cells from cam2 and cam2CΔ$^{-/-}$ mice (*below*). The stainings show that bone marrow and splenic B220$^+$ cells from mice carrying the dromedary H chain transgene express HEL-specific Ig not accompanied by endogenous IgM or L chain.

camCΔ$^{-/-}$ mice, which established that the dromedary H chain transgene promotes B cell development independent of μ or expression of other endogenous H and L chains. Expression of HEL-specific H chain Abs on the cell surface suggests H chain association without L chain in dimeric or multimeric form. Such association presents a new type of BCR, with the capacity to initiate B cell development independent of conventional Ig expression.

Endogenous H and L chain loci are rearranged and transcribed

The lack of endogenous Ig expression suggested that the H chain BCR elicits the appropriate signals to prevent endogenous rearrangements. However, when analyzing bone marrow and spleen cell DNA from cam2 mice by semiquantitative PCR, we found, apart from the expected D-J_H bands, similar levels of V_HDJ_H and VκJκ rearrangement in cam2 and normal mice (Fig. 7). Using forward oligos representing different V_H gene families, *J558* and *7183*, gave a clear indication of diverse rearrangements in the cam2 mice, but we cannot completely rule out that endogenous V(D)J rearrangements are detected in B cells that have lost transgenic expression of the dromedary H chain. Hybridization with a full-length *J558* V_H gene further confirmed the similar levels of VDJ rearrangement in cam and normal mice. The obtained PCR fragments were of the expected sizes (3, 35). In the experiments, DNA from RAG2$^{-/-}$ tissues, used as a negative control, showed some background amplification. Such background was not seen when, for example, mouse embryonic stem cell DNA was used for V_HDJ_H amplification (data not shown). Different RAG2$^{-/-}$ mice sources and DNA preparations did not prevent this but always showed a much reduced signal compared with cam2 and normal mouse DNA. However, because we did not see any background in RT-PCR using RAG2$^{-/-}$ RNA, this could indicate a low level of nonproductive recombination products. Using lamin B1 as reference (3) for the semiquantitative comparison using serial dilutions, we found no indication of reduced levels in the cam mice. Control reactions conducted in parallel using normal mouse DNA for dromedary H chain amplification and dromedary *V_HH-Cy2aTM* plasmid DNA for mouse V(D)J PCR did not result in nonspecific bands (data not shown).

To our surprise, RT-PCR signals, reflecting RNA levels, were also very similar in cam2 and normal mice. To assess whether the V558-J_H RT-PCR bands from cam2 mice accounted for nonfunctional VDJ rearrangements or represented potentially productive transcripts, we cloned and sequenced the ~400-bp fragments (supplemental Table II). Sequence comparison established that fully functional and diverse murine V_HDJ_H transcripts were produced in cam2 mice. To investigate whether endogenous transcripts were only expressed in cells that did not produce dromedary H chain Ig or whether endogenous and exogenous transcription was jointly operative in the same cell, we separated B220$^-$ and B220$^+$HEL$^+$ lymphocytes by flow cytometry (Fig. 8). Semiquantitative RT-PCR analysis of B220$^+$HEL$^+$ bone marrow and spleen cell RNA from cam2 mice showed extensive V558-J_H and Vκ-Cκ amplification similar to those from normal mice. In B220$^-$ cells, V(D)J transcripts were also well maintained, and certainly in the cam mice, there were no amplification differences. As this raised the possibility that the calculated purity, >95%, of the sorted cell populations may not have been reached, we used further RT-PCR to identify surrogate L chain transcripts. With VpreB and λ5, surrogate L chain polypeptides are well expressed in B220$^+$ bone marrow cells, but no expression is found in the spleen (Refs. 9, 11, 42, and refs. therein). This was exactly what we found and provided reassurance of the purity of the analyzed cell populations. A comparison of sorted B220$^+$ and B220$^-$ bone marrow and spleen cells from cam2 and normal mice showed no difference in transcription levels of the surrogate L chain (Fig. 8). Expression of surrogate L chain in bone marrow but not spleen B220$^+$ lymphocytes from cam mice was independently confirmed by cytoplasmic staining with anti-λ5 (data not shown). Control reactions (Fig. 8*C*) using cDNA prepared from bone marrow and spleen cells of RAG2$^{-/-}$ mice, bone marrow cells from SL (surrogate L chain triple knockout (KO))$^{-/-}$ mice (42), and DNA from normal mouse spleen cells confirmed the validity of the RT-PCR. The lack of VpreB and λ5 transcription in mature cam2 B cells rules out that dromedary H chain expression relies on the presence of surrogate L chain.

Our comprehensive analysis of intra- and extracellular expression of endogenous murine Ig revealed that very small amounts, if any, were retained in the cell. This may mean that either no translational products were being produced or that there was rapid degradation. Staining with anti-L chain confirmed a lack of endogenous Ig. In addition, we did not identify dromedary H chain transcripts in other nonlymphocyte tissues (data not shown). In

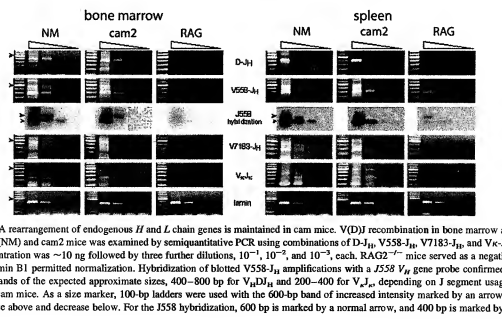

FIGURE 7. DNA rearrangement of endogenous *H* and *L* chain genes is maintained in cam mice. V(D)J recombination in bone marrow and spleen cells from normal mice (NM) and cam2 mice was examined by semiquantitative PCR using combinations of D-J_H, V558-J_H, V7183-J_H, and Vκ-Jκ primers. The DNA starter concentration was ~10 ng followed by three further dilutions, 10^{-1}, 10^{-2}, and 10^{-3}, each. RAG2$^{-/-}$ mice served as a negative control, and amplification of lamin B1 permitted normalization. Hybridization of blotted V558-J_H amplifications with a *J558* V_H gene probe confirmed the specificity of the reactions. Bands of the expected approximate sizes, 400–800 bp for V_HDJ_H and 200–400 for $V_\kappa J_\kappa$, depending on J segment usage (3, 35), were maintained in the cam mice. As a size marker, 100-bp ladders were used with the 600-bp band of increased intensity marked by an arrow, followed by a 100-bp size increase above and decrease below. For the J558 hybridization, 600 bp is marked by a normal arrow, and 400 bp is marked by a small arrow.

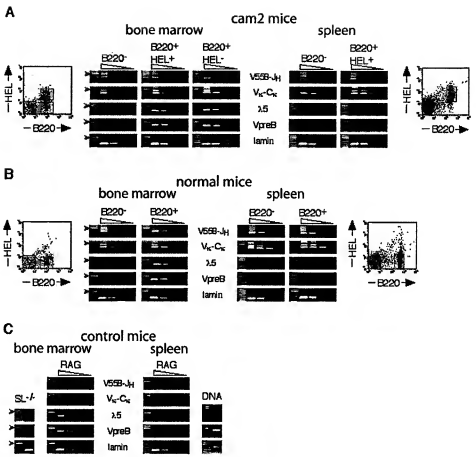

FIGURE 8. V(D)J transcription is operative in cam mice. Bone marrow and splenic lymphocytes from cam2 and normal mice, stained for B220 and HEL binding, were sorted by flow cytometry to >95% purity. RNA was produced from 10^5–10^6 sorted cells, and equal amounts of cDNA from ~5×10^3 cells were analyzed in parallel by PCR in successive 10-fold dilutions. Oligo combinations of V558-J_H and V_κ-C_κ determined the level of Ig transcription, λ5 and VpreB examined the developmental stage of the cell populations, and lamin provided an independent reference of the cDNA concentrations. *A*, RT-PCR analysis of gated bone marrow and spleen cell populations, $B220^-$, $B220^+$, and $B220^+HEL^+$, from cam2 mice. *B*, $B220^-$, $B220^+$ cells from normal mice. *C*, Control reactions using RT-PCR analysis using tissue from surrogate L chain KO mice (42) and $RAG2^{-/-}$ mice, and PCR using normal DNA, which confirmed the size difference of the genomic products.

conclusion, this suggests that cam transgenic mice express the dromedary H chain-only Ab without association or attendance of significant amounts of endogenous Ig. Nevertheless, it may be possible that the transgene can only be expressed after endogenous V(D)J rearrangement has been completed. However, dromedary H chain expression in $C\lambda^{-/-}$ mice, which do not express rearranged H chains without C region (3), rules out that B cell differentiation is driven by endogenous IgH expression. The results show that endogenous H and L chain genes, despite being fully rearranged and transcribed, are excluded from expression at the translational stage by a feedback signal originating from a H chain BCR without L chain.

Discussion

Introduction of a rearranged dromedary *H* chain gene into the mouse germline showed that Ag-specific H chain-only Abs could be correctly expressed, without the C_H1 domain, and assembled as multimer. H chain Ig was secreted and also presented on the cell surface, which led to progression in B cell development. Expression of H chain IgG might exclude translation of endogenous H and L chain polypeptides, which established a BCR without L chain association.

The rearranged H chain expressed in transgenic mice was constructed with no alteration that would favorably bias expression in mouse B cells. Thus, secretion and surface expression of HEL-specific H chain Abs in a heterologous system established that RNA processing, H chain assembly, and cellular transport use commonly recognized signals provided by the dromedary V_HH-γ2aTM construct. The likely reason why dromedary H chain Ig can be expressed in the mouse seems to be due to two gene adaptations in camelids, not found in other jawed vertebrates. Their V_HH genes are distinct from conventional V_H genes; they accommodate changes in key residues normally in contact with the V_L domain in the Ag binding site of conventional Abs (16). Apparently, neither the V_HH hallmark amino acids, nor the presence of a long CDR3 loop of 24 aa, caused folding problems (43). Nevertheless, the genomic organization of the V_HH genes (i.e., promoter, leader signal, intron, V-exon, and recombination signal sequence) is otherwise remarkably similar to that of the conventional V_H counterparts (44). It has been reasoned that V_HH genes have recently evolved from conventional V_H genes after the emergence of the Tylopoda (>50 million years ago), which makes it likely that both types are accommodated in the *V* gene cluster of the *H* chain locus (44). This is supported by the observation that both the V_H and V_HH gene segments appear to rearrange to the same *D* and J_H gene segments to form either a conventional Ab or a H chain Ab (21). The other adaptation concerns a subset of their Cγ genes (24, 25). It was proposed that in these genes, a point mutation at the canonical splice signal sequence might cause the excision of the first C region domain (24). Although the precise mechanism is not known, this removal seems to permit assembly and secretion of homodimeric H chains (20). Interestingly, accurate and highly efficient removal of the C_H1-containing sequence from the RNA transcript of *H* chain genes appears to be performed with equal efficiency in camelids and transgenic mice. Neither in the dromedary nor in our transgenic mice could γ2a *H* chain genes with retained C_H1 exon be identified by RT-PCR and sequencing. Thus, the removal of the C_H1 exon appears to be essential to permit expression of H chain Ig. However, exclusive H chain-only Ab production in camelids was predicted to involve interaction with

species-specific cellular factors important for the expression of H chain Ab genes, processing of their transcripts, and the assembly of the translation products into functional Ag binding entities (45). For this reason, it was unexpected to see that a heterologous system produced functional H chain Abs at quite respectable protein levels. This suggested that intrinsic alterations of the dromedary H chain Ab are well recognized and dealt with by the mouse B cells and that dromedary-specific factors are either not essential or can be bypassed by the mouse transcription, translation, and secretion machinery.

The C_H1 domain participates actively in the regulation of the assembly and secretion of conventional H2L2 Abs via association with BiP (26–29). A lack of C_H1 is likely to permit unhindered transit of the H chain polypeptide through the endoplasmic reticulum to allow secretion and appropriate surface deposition. Furthermore, the loss of BiP association may also prevent degradation of the H chain. H chains with the long hydrophobic transmembrane region anchor in the lipid bilayer, whereas the short hydrophilic C-terminal region of secretory form H chains ensures their release from the cell in the absence of associated BiP. The importance of the C_H1 domain is well recognized because hybridoma or myeloma cell lines harboring Ig genes with deleted C_H1 exon retain the ability to secrete homodimeric H chains without associated L chains (46, 47). In heavy chain disease, truncated H chains are readily secreted without L chain (17, 48). For the dromedary H chain, not being dependent on IgM expression may allow the expansion of a different lymphocyte subset, which may be able to restore normal B cell development. Extensive levels of $B220^+$ cells, some with dendritic cell characteristics, have been found in bone marrow and spleen (49) and may be maintained in the dromedary H chain mice. Alternatively, expression of the rearranged dromedary H chain gene could facilitate progression in B cell development to a mature stage without the differentiation stages from pro- to pre-B cells ($B220^+CD43^-$ cells in Fig. 5). In this context, it is notable that staining of camel lymphocytes for Ig H and L chain on the cell surface has been attempted but did not unambiguously demonstrate surface IgG H chain-only expression. A reason for this may be that the staining reagents raised against ruminant Ig fail if there is broad epitope diversity (50). Despite this setback, camelids readily produce Ag-specific Abs in H2 and H2L2 configuration, and there is no indication that mixed molecules are expressed (16, 51). Unfortunately, there is no information about pre-B cell development in camelids or whether an H chain without C_H1 can associate with a surrogate L chain to form the pre-BCR necessary to progress B cell development. However, from gene targeting studies in the mouse, it is clear that B cell development without surrogate L chain can progress (42), whereas B cell development without L chain is blocked after H chain expression and maturation up to the immature B cell stage (4).

The various Ig classes seem to form distinct oligomeric BCR complexes, which may differ in their threshold levels for BCR signaling (Ref. 52, and refs. therein). For example, the IgG BCR complex, in contrast to the IgM or IgD BCR complex, cannot give an efficient positive selection signal. Perhaps, contradictory to expectation, the H chain BCR may be able to provide an adequate differentiation and proliferation signal to secure survival. In transgenic mice carrying rearranged conventional *H* chain genes (μ, δ, γ, or α), feedback inhibition can prevent DNA rearrangement of the endogenous *IgH* locus (References 53–55, and references therein). However, the expression of the transmembrane form of introduced Ig transgenes does not necessarily prevent DNA rearrangement of the endogenous loci to secure allelic exclusion (56–58). It has also been shown that *γ2b* transgenes are coexpressed with endogenous μ, and that *γ2b* cannot by itself promote B cell development in the μ KO background (40, 55, 59). The few mature B cells that do develop in the transgenic mice express both endogenous μ and transgenic γ2b, and in addition, L chain is expressed. Although these experiments show that B cell development is critically dependent on signaling of a μ-H chain associated as BCR, there are exceptions. In a particular *γ2b* transgenic mouse line, it appears that transgene expression by itself can promote B cell maturation and allelic exclusion, possibly by expanding a particular B cell subset (55). In separate founders, most likely carrying a γ2b transgene integration at diverse chromosomal locations, it was discovered that alternative expression pathways were used, maybe dictated by different expression levels. Despite these contradictory results, which may largely depend on site of integration and copy number of particular *IgH* transgenes, the overall conclusion from transgenic IgH mouse studies is that B cell maturation can progress, but the developmental state of the lymphocytes appears to be critically dependent on the onset of (endogenous) μ expression. This was not seen in the cam mice, where even in a normal mouse background, IgM expression was prevented without causing developmental cessation.

The two cam transgenic lines we describe in this study, derived from independent microinjections, are most likely to carry the transgenes at different chromosomal sites. Despite this and the low H chain expression level in cam1 mice, developmental progression is very similar. The recent finding that, in the mouse, entire μ-H chains can be transported and expressed on the surface of pre-B cells without associated L chains (9–11) contradicts previous discussions that free H chain polypeptides are toxic and that they have to be neutralized to allow progression in B cell development (Ref. 60, and refs. therein). The observations may be compatible if lower H chain expression levels in early B cells are taken into account and if apoptosis, which may be induced when insoluble (accumulated) H chain complexes damage the cell, occurs at a later differentiation stage. The lack of L chain in the cam mice must be the result of the failure of the dromedary H chains to associate with L chains, which arises from the difference in important residues in V_HH genes compared with V_H genes (44). Prohibited L chain association may act as a feedback signal that stops L chain translation. This would be in agreement with the observation that in healthy individuals, H chain expression balances L chain synthesis to accomplish equimolar levels (61). In the dromedary H chain mice, which are perfectly healthy, the induced lack of L chain may prevent expression of endogenous H chains, which, if not removed, could be toxic for the cell. In addition, this emphasizes that the introduced dromedary H chain appears to be fully active in securing allelic feedback, albeit at the very late translational stage, which still allows productive DNA rearrangements and transcription of potentially functional endogenous H chains.

The presence of the BCR is essential to govern B cell survival and differentiation (62). Thus, H chain Ab deposition on the cell surface is of key importance for the formation of the H chain Ab repertoire (63, 64). The formation of H chain Abs in camelids is decided by rearrangement of a V_HH gene to commonly used D and J_H segments (44) and (switch?) recombination to a *Cγ* gene that permits the removal of C_H1 (22, 24). We speculate that transitory surface expression of μ-H chain without L chain association, as described in the mouse (9–11), may also occur in camelids, and perhaps, unlike in mice, may facilitate successful switching and expression of H chain IgG isotypes with their particular *V* genes, which do not tolerate association with L chain (15, 16, 23). Expression of the membrane form strongly suggests the presence of memory B cells for H chain Abs in camelids. Such cells would undergo an Ab maturation process, leading to H chain Abs with

improved affinities for the Ag. The finding of extensive diversification of H chain Abs (21), but the failure to detect an IgM isotype without L chains in camelids (22, 23), has unexpected implications for H chain Ab ontogeny because it questions the involvement of μ^+ B cells bearing conventional IgM as precursors of H chain Ab-producing cells. For this reason, it becomes important to reassess the developmental progression of B lymphocytes, which can express H chain Abs. The successful generation of transgenic H chain Abs paves the way for the creation of single-chain repertoires in the mouse by introduction of modified V_H genes and splice site mutation in a γ-H chain gene. Retention of V gene variability allows recognition of novel epitopes regarded as poorly accessible by conventional Abs and provides the advantage that single-chain binders are not dependent on successful V_H/V_L pairing (16).

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Disclosures

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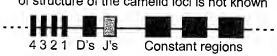
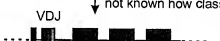
immunizecamelid with a specific antigen

VHH's embedded among VH segments, rest of structure of the camelid loci is not known



VDJ rearrangement e.g. VHH3, it is not known how class switching occurs



hypermutation in periphery, i.e affinity maturation for specific antigen and camelid class switching

transcription in camelid B cells

cDNA

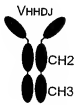
RNA

translation in camelid B cells

cloneVHHDJ in expression vector

introduce into transgenic mice

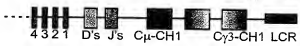
transcription and translation in transgenic mouse B or other cells yields a camelid VHHDJ (no effector CH2/CH3) same sequence as in the camelid cDNA VHHDJ above



HCAb from camelid

LEDBETTER

heavy chain only camelid antibody diversity and selection for specific antigen response carried out in the camel or llama



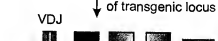
generate a (human) immunoglobulin locus with VH segment, D segments,J segments and constant regions lacking CH1

introduce into transgenic mice preferably without a functional endogenous murine immunoglobulin heavy chain locus, e.g μMT

transgenic mouse

immunize

VDJ rearrangement (e.g. VH4) of transgenic locus



hypermutation in periphery, i.e affinity maturation for specific antigen and class switching (e.g. to Cγ2)

transcription in mouse B cells

RNA

translation in mouse B cells

newly generated (human) HCAb (IgG)

THIS PATENT (JANSSENS ET AL)

heavy chain only (any species) antibody diversity and selection for specific antigen response carried out in transgenic mouse

1IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Frank Grosveld	**Confirmation No. 9062**
Serial No.: 10/692,918	**Art Unit: 1632**
Filing Date: October 24, 2003	**Examiner: Anoop K. Singh**
For: IMMUNOGLOBULIN 2	
Customer No.: 34132	

DECLARATION OF RUDOLF GROSSCHEDL, Ph.D.

1. I have been engaged by Erasmus University to provide my opinion on certain issues concerning the above-identified application. I am being compensated at a rate of $ 360 per hour to do so.
2. My short form *curriculum vitae* ("cv") is attached as Exhibit A.
3. I received a Masters of Science in biology from the University of Freiburg, Germany, in 1978. I received a Ph.D. in molecular biology from the University of Zurich, Switzerland, in 1982. I served as a Postdoctoral Fellow in the laboratory of Dr. David Baltimore, the Whitehead Institute and Massachusetts Institute of Technology, Cambridge, Massachusetts.
4. I am currently the Director, Max-Planck Institute of Immunobiology, Department of Cellular and Molecular Immunology, Stubeweg 51, 79108 Freiburg, Germany.
5. As Director of the Max-Planck Institute of Immunobiology, my duties include supervising research, hiring and mentoring of junior group leaders
6. As is evident from my cv, I have been involved in research regarding the expression of immunoglobulin genes and transgenic animals since at least 1988, and have published extensively on the topics. More specifically, I have been involved in research concerning tissue-specific expression of immunoglobulin genes in transgenic mice. I am on the editorial boards of three peer-reviewed journals – *Molecular and Cellular Biology, Genes and Development, and Immunological Reviews.*

7. I have reviewed the above-identified application, the correspondence with the examiner and the recent response from the inventor Professor Grosveld. I am familiar with the involved technology, namely single heavy chain antibodies. Single heavy chain antibodies are antibodies composed of heavy chains only; no light chains are present.

8. All claims of the above-identified application are currently under rejection by the U.S. Patent & Trademark Office ("the Office"). I have read the Final Rejection dated February 12, 2007. I have also read the Interview Summary dated June 1, 2007; the Request for Reconsideration ("Request") filed June 25, 2007; and the Advisory Action Before the Filing of an Appeal Brief ("Advisory Action") dated July 9, 2007.

9. In the Final Rejection, the Office rejected the claims under the following bases:

    1. For lack of enablement under 35 USC § 112, first paragraph;
    2. For being incomplete as omitting essential steps under 35 USC § 112, second paragraph;
    3. As anticipated by Ledbetter et al (WO 99/42077) under USC § 102(b); and
    4. For obviousness-type double patenting over the claims of co-pending Application No. 10/692,918.

    I have been asked to address basis 1 above. The Office's view is that undue experimentation would be required to practice the invention. I respectfully disagree. The first basis for rejection is further broken done into four issues. Based on my expertise, I will be addressing the third and fourth issues.

10. The third issue is that the claims cover the use of any promoter, with any regulatory elements, in any non-human transgenic mammal. The Office alleges that, at the time of invention (which I understand to be at least April 24, 2001, the earliest priority date claimed), only the mouse was recognized by the art of record as a routinely manipulated animal, and that the art of record recognized the unpredictability of making transgenic animals other than mice (see page 8 of the Final Rejection). I do not agree that the art of record shows this.

11. It is well established that large tracts of human genomic sequence can be transferred into the mouse or other mammalian genomes using YACs. Moreover, human genes and regulatory elements when placed in a mouse or another mammalian background respond to tissue specific and developmental signals as would the endogenous homologue. This is

particularly well documented for the human immunoglobulin heavy and light chain genes which respond in a mouse background to antigen challenge in a B-cell specific manner leading to the production of human immunoglobulins (see Jacobovits, A (1994) Current Biology, 4, 761-763 and references cited therein) **(copy attached)**. Human heavy and light immunoglobulin gene loci have also been expressed as transgenes in cattle (Kuroiwa et al., *Nat Biotech.*, 2002; 20:889-894, "Cloned Transchromosomal calves producing Human Immunoglobulin" **(copy attached)**. Thus the mouse provides a valid model to evaluate gene expression with the expectation that if a gene is functionally expressed in the mouse it will also be expressed as a transgene in other non-human mammals. Thus for expression of a camelised human immunoglobulin heavy chain only gene loci the same YAC cloning strategy reviewed by Jacobovits has been followed, excepting that the final loci used are devoid of CH1, and comprise selected V gene segments (in Janssens et al Llama VHH-segments, and in the recent data disclosed by Professor Grosveld in his declaration that I understand is being submitted concurrently - human VH segments). All regulatory elements known to ensure high levels of expression in B-cells are present in the YAC construct used in the specification as described in the prior art. In particular, the enhancer element required for the initiation of the recombination and expression of the IgH locus is located in the intron between the JH and Cμ regions (Gillies et al., 1983, Grosschedl and Baltimore, 1985; Sen and Baltimore 1986, and references cited therein; **copies attached;** *see also* Serwe and Sablitzky (1993), cited on page 23, lines 10-11 of the specification), while the enhancers (LCR) that ensure high levels of the rearranged IgH transcripts regardless of position are located to the 3' side of the last constant region (mouse locus, Pettersson et al., 1990; human locus, Mills et al 1997; **copies attached**). Use of both the intronic enhancer and the 3'LCR was known to confer correct spatial and position-independent expression of a linked transgene in mice (Grosschedl et al., 1984; Lieberson et al., 1995; Chauveau et al., 1999; Janssens et al, 2006; **copies attached**). Both sets of elements were present on applicant's llama-human IgH locus. Briefly, applicant took PAC clone 1065 N8 (BABPAC Resource Center Oakland CA) containing the intronic enhancer sequences and Bac clone 11771 (Incyte Genomics, PaloAlto CA) containing the LCR. The intronic enhancer region was cloned into the final construct as a 120kb SalI fragment (i.e. similar

to Bruggemann et al., Figure 1 top line). The LCR was cloned as a combination of three fragments, BclI/BamHI (3.7kb containing HS1), BamHI (8kb containing HS1 and 2) and BamHI/BglII (7.5 kb containing HS4) into BlueScript and transferred to the final construct as a NotI/SalI fragment. The LCR sequences are the same as shown by Mills et al (page 846 Figure 1).

12. The presence of the Ig LCR is preferable but not essential. As noted above, its presence ensures that every transgene is transcriptionally active irrespective of its site of insertion in the host genome, an important consideration when working with larger mammals, such as cattle and sheep, with long breeding cycles relative to the mouse (Grosschedl et al., 1984; Lieberson et al., 1995; Chauveau et al., 1999), but not necessary for smaller mammals.

13. Since the regulatory elements present in an Ig heavy chain transgene comprising CH1 (in this instance human) determine B-cell specific expression in mice and cattle, then the introduction of a heavy chain only immunoglobulin loci (devoid of CH1) comprising the same regulatory elements into a non-human mammalian host should also result in the expression of the introduced transgene, the observed result in mice. Consequently if a human IgH locus devoid of CH1 was introduced into cattle, or for that matter any non-human mammal, it would be reasonable to expect based on the evidence available that the IgH gene (devoid of CH1) would be expressed and human heavy chain only antibody would be present in the serum. This conclusion is supported by Buelow and others who have published on the expression of human and humanised immunoglobulin genes in non-human mammalian species as diverse as rabbit, pig, sheep, and cow (see WO 02/12437 A2 and references therein, **copy attached**). Thus the elimination of CH1 domains from these immunoglobulin genes would predictably result in the production and circulation of heavy chain only antibody as observed by Janssens et al in transgenic mice.

14. Experimental conditions for the introduction of genes into the germ-line (transgenesis) have been optimised for rodents and other laboratory animals, also many animals of agricultural importance e.g. rabbit, pig, goat, sheep, cattle. Hence, the application of this technology as described and applied to mice could be followed by someone of ordinary skill in the art of the derivation of heavy chain only antibody in non-human transgenic

animals, following the guidance provided in the specification.

15. There is no scientific evidence to suggest that natural IgH regulatory elements active in one non-human mammal will not be active in another. In fact, the IgH regulatory elements have been well characterized, and I am not aware of reports in which a gene that is expressed faithfully in one mammal is not expressed in another mammal. Rare cases, in which poor expression has been reported, do not reflect the field and are typically due to the use of incomplete constructs.

16. The final issue of the first basis for rejection focuses upon the insertion of the vector into the mammals. Although acknowledging that insertion of the vector can be accomplished by either microinjection (into fertilized eggs) or through use of embryonic stem (ES) cells, the Office states that the method requires the use of ES cells, which the Office then states is restricted to mice (page 10 of the Final Rejection). Janssens et al., however, reports that the vectors were injected into fertilized mouse eggs, not ES cells. Use of ES cells, thus, is not required. I would expect that any route of introduction of the vector into the germ-line of any mammal would result in heavy chain antibody production by B-cells.

17. Based on the overwhelming evidence available it is not unreasonable to conclude that the invention (methods for the in vivo derivation of heavy chain only antibodies in transgenic non human mammals in response to antigen challenge) using the natural mammalian IgH regulatory elements as set out could equally be applied by those skilled in the art for the derivation of heavy chain antibody (devoid of CH1) as a result of antigen challenge in any non-human mammalian background in addition to the mouse, for example rat, rabbit, pig, goat sheep, cow, monkey, etc. Indeed, the inventor used human regulatory elements in the mouse with success. Thus, the route described for the derivation of heavy chain only antibody as exemplified in the application and by Janssens et al using the mouse, can be equally applied to any non-human mammalian system. Specifically, I note the following.

- The introduction of transgenes into the germ-line of non-human mammalian systems was well established at the time of filing.
- Regulatory elements that ensure high levels of B-cell specific IgH were well characterised and were known to be present in the human IgH genomic sequence

described, moreover these were known to be functional when incorporated in transgenes in non-human mammals at the time of filing.

- At the time of filing, there was a wealth of evidence to demonstrate that enhancer and LCR elements derived from one mammalian species (including human) are functional in other mammalian species.

18. I hereby declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2/3/08 Rudolf Grosschedl

Date Dr. Rudolf Grosschedl

CURRICULUM VITAE

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EDUCATION

1971-1972 University of Salzburg, Austria

1972-1978 University of Freiburg, Germany
M.S. in Biology, Thesis topic: Structural analysis of the replication region of lambdoid bacteriophages
Thesis advisor: Dr. Gerd Hobom

1978-1982 University of Zurich, Switzerland
Ph.D. in Molecular Biology
Thesis topic: Functional analysis of an histone gene promoter
Thesis advisor: Dr. Max L. Birnstiel

RESEARCH EXPERIENCE

1977-1978	Undergraduate Student, Laboratory of Dr. Gerd Hobom University of Freiburg, Germany
1978-1982	Graduate Student, Laboratory of Dr. Max Birnstiel University of Zurich, Switzerland
1982-1985	Postdoctoral Fellow, Laboratory of Dr. David Baltimore Whitehead Institute and Massachusetts Institute of Technology Cambridge, Massachusetts, USA

POSITIONS HELD

1982-1985	Postdoctoral Fellow Massachusetts Institute of Technology Cambridge, Massachusetts, USA Advisor: Dr. David Baltimore
1986-1992	Assistant Professor Department of Microbiology and Immunology University of California, San Francisco
1988-1992	Assistant Investigator Howard Hughes Medical Institute San Francisco, California, USA
1992-1995	Associate Professor Department of Microbiology and Immunology Associate Investigator Howard Hughes Medical Institute University of California, San Francisco
1995-1999	Professor and Vice Chair Department of Microbiology and Immunology Investigator Howard Hughes Medical Institute University of California, San Francisco
1999-2004	Professor and Director Institute of Biochemistry and Gene Center University of Munich, Germany
2004-present	Director Max-Planck-Institute of Immunobiology Freiburg, Germany

AWARDS

1982	Postdoctoral Fellowship from the European Molecular Biology Organization
1985	Special Fellowship from the Leukemia Society of America
1987	Leukemia Society of America Scholar
2000	Elected Member of the European Molecular Organization

ADVISORY BOARD

1999-2003	*Atugen AG*

EDITORIAL BOARDS

1990-present	*Molecular and Cellular Biology*
1992-present	*Genes and Development (European Editor 2002 – 2004)*
2002-present	*Immunological Reviews*

RESEARCH TOPICS

Lymphocyte differentiation, gene regulation, Wnt signaling

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Humanizing the mouse genome

The ability to replace mouse genes with their human equivalents using 'yeast artificial chromosome' technology provides a powerful new technique for studying the regulation and function of human genes.

The current human genome initiatives, which aim to map and ultimately sequence the complete human genome, are expected to produce a wealth of information about the chromosomal location and physical organization of many human genetic loci. However, while such studies can assign the transcribed region of a gene to particular DNA fragments of known position within a physical map of the human genome, by themselves they cannot fully define the extent of a gene — which includes regulatory sequences that can be some distance from the transcribed region — nor can they identify the gene's biological functions (though in some cases there will be hints from human mutations or sequence similarities to genes of known function). Thus, it is important that strategies and techniques are developed that will enable the functions of cloned human DNA sequences to be identified, preferably in a whole-animal context.

Ideally, such techniques will allow large stretches of human DNA to be transplanted into a 'model' mammalian host, in which the corresponding genes have been inactivated so that the host is dependent upon complementation of their biological functions by the introduced human genes. At present, the preferred host for such a strategy is the mouse, a small, prolific mammal that is amenable to genetic manipulation and that has been the subject of extensive genetic analysis for many years. Mouse embryonic stem (ES) cells have proved to be an effective tool for introduction of defined and selected genetic modifications into the mouse germline. When integrated into the mouse genome in ES cells, large DNA inserts can be evaluated for structural integrity, stability, copy number and integration into active chromatin sites, prior to their transmission into mice.

The development of yeast artificial chromosome (YAC) technology has permitted the cloning and genetic modification of DNA segments thousands of kilobases in size [1]. Such molecules are too large to be cloned by conventional cloning technologies, but when cloned between the YAC vector arms (Fig. 1) they can be stably maintained in yeast and are amenable to genetic manipulation by homologous recombination, which occurs efficiently in the host yeast cells.

YACs can be introduced into ES cells simply by isolating them from yeast cells and transfecting the ES cells with purified YAC DNA fragments. With YACs that are particularly large — as would be required to transmit complex human loci in intact form — however, there is a danger that purification would shear the YAC DNA. This danger can be avoided — and the chances of preserving the structural identity of the YACs consequently increased — by an alternative approach, in which the YACs are shuttled into ES cells by fusion with yeast spheroplasts, shown previously to work with mammalian cell lines [2]. Questions can be raised about the effects of the fusion process itself, and the presence of co-transferred yeast genomic sequences, on the ability of ES cells to differentiate properly and to recolonize the mouse germline, but, as explained below, recent results suggest any such effects are negligible.

The faithful delivery of YACs carrying large fragments of human DNA into the mouse germline, using the cell fusion technique, was first reported last year [3] with a 670 kilobase (kb) YAC carrying a human X-chromosome fragment. The human hypoxanthine phosphoribosyl transferase (HPRT) gene, which occurs naturally on the human X-chromosome fragment carried by the YAC in this experiment, was used as a selectable marker following the polyethylene glycol-mediated fusion of HPRT-deficient ES cells with YAC-containing yeast spheroplasts. All the clones selected for expression of the HPRT gene were shown to contain the YAC, most (>90%) retaining the human insert completely intact, unrearranged and in a single copy.

About 40% of the selected clones retained both vector arms, indicating that the entire 670 kb YAC had integrated into a mouse chromosome and suggesting that the vector arms could be used to carry markers for the selection of transformed ES cells. The co-integrated yeast

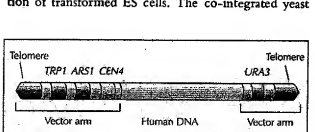

Fig. 1. Outline of the organization of a yeast artificial chromosome (YAC), which can be used to transfer large fragments of human DNA into the genomes of mouse ES cells and from there into mice. Both vector arms end in yeast telomeres, and one carries a centromere (*CEN4*) and a replication origin (*ARS1*); two genes (*TRP1* and *URA3*) act as selectable markers that stabilize the YACs in yeast cells.

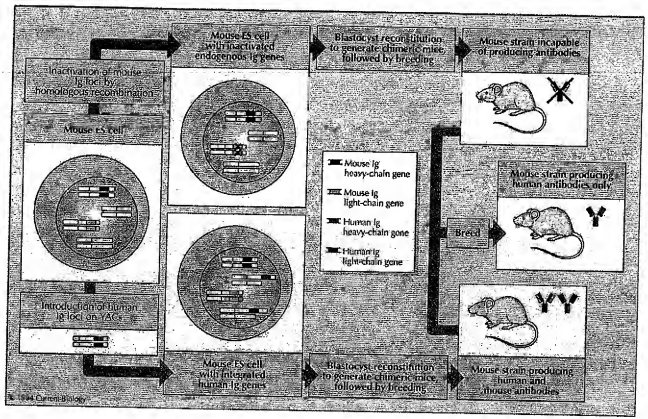

Fig. 2. An outline of the strategy my colleagues and I have recently used [8] to generate mice expressing human Ig genes, introduced on YACs, but not endogenous mouse Ig genes, inactivated by homologous recombination in ES cells.

genomic sequences did not affect the ability of ES cells to differentiate properly, either *in vitro* or *in vivo*, or to give rise to mice carrying the intact human X-chromosome fragment in their germline. The transmitted human sequences retained their function in the transgenic mice, as shown by the expression of human HPRT in all mouse tissues tested.

An alternative strategy for introducing YACs into ES cells is by transfecting the cells with gel-purified YAC DNA carried in lipid micelles, a technique known as lipofection. The feasibility of this approach has been demonstrated [4] with a 150 kb YAC encompassing the mouse $\alpha 1(I)$-collagen gene and flanking sequences, and also carrying a selectable neomycin-resistance gene. In 10% of the selected ES clones, the entire YAC was found to be intact and unrearranged. The YAC was transmitted faithfully into the mouse germline and the collagen transgene was found to be expressed at levels similar to that of the endogenous gene.

The demonstrations [3,4] that these two techniques — cell fusion and lipofection — both allow the efficient and reliable transfer of YACs into the mouse germline via ES cells has opened the way for their use to study the function and regulation of large and complex human genes, such as the β-amyloid precursor protein (APP) locus and the immunoglobulin (Ig) gene loci. The human APP gene — 400 kb long, composed of 18 exons and located on chromosome 21 — has been suggested to play a role in the pathogenesis of Alzheimer's disease and Down's Syndrome. In the hope that models for these human disorders might be developed from mice expressing the human APP gene in a properly regulated manner, two groups [5,6] have independently used the lipofection approach to transfer into ES cells a 650 kb YAC carrying the entire human APP gene and flanking sequences, and have generated mice with the intact locus in their germline. Human APP transcripts [5,6]) and proteins [5], with expression patterns mirroring those of endogenous mouse APP, were detected in the brain and peripheral tissues of these transgenic mice. The fidelity with which the human APP locus is regulated in the transgenic mice suggests that they may serve as a model system for studying the nature and development of Alzheimer's disease, and for testing therapeutic approaches to its prevention.

The genes encoding human Ig heavy and light chains each span over 2 megabases. In their germline configuration, these genes consist of separate segments encoding distinct parts of the Ig chains, such as the *V D* and *J* segments that encode the variable domains, and the *C* segments that encode the constant domains. The gene segments are sequentially joined by DNA rearrangements that accompany the development of mature B cells [7].

The ability to transfer these loci to the mouse genome using YAC technology has made it possible to assess the compatibility of human sequences with the mouse Ig gene recombination and expression machinery, and to study the relationship between the content and organization of the human sequences and their tissue-specific expression and function. Using the recently developed [8] strategy outlined in Figure 2, it is now possible to test the ability of human loci to substitute for their mouse equivalents — and in particular to generate mice that make fully human antibodies, which have a lower immunogenicity in humans and more desirable pharmacological properties than engineered mouse antibodies, and so are more suitable for human therapeutic applications.

Human genomic DNA fragments containing germline configuration *V* and *C* gene segments, including regulatory elements, of the Ig heavy-chain [8,9] and κ light-chain [8,10] loci have been successfully introduced into mouse ES cells using both fusion [8,10] and lipofection [9] techniques. The transformed ES cells were used to generate mice expressing individual human immunoglobulin genes [8,10]. In our work, the fusion technique worked with high efficiency, yielding a high frequency of clones carrying a single intact YAC [8]. A significant number of ES clones bearing an intact YAC but lacking detectable yeast DNA sequences were identified [8,10]; when present, however, the co-integrated yeast genomic DNA did not interfere with proper mouse development or with the assembly and expression of the human Ig genes [8].

The human Ig gene sequences were recognized, irrespective of their copy number or integration site, by the mouse machinery for gene recombination and expression. This was shown by their ability to undergo diverse rearrangements [8,10], to be expressed at significant levels and to prevent rearrangement of the mouse Ig genes (equivalent to the 'allelic exclusion' that normally ensures that B cells express only one Ig heavy-chain and one light-chain gene) [8]. In mice carrying the two types of human Ig YAC, both heavy and light Ig chains were expressed in B cells and assembled to generate membrane-bound or secreted, fully human antibodies [8]. When expressed in mice in which both endogenous heavy and κ light-chain genes had been inactivated by homologous recombination (Fig. 2), the human Ig genes were again rearranged and expressed properly [8], restoring B-cell differentiation and maturation. The treatment of these transgenic mice with antigen elicits an antigen-specific, human antibody response [8].

The human Ig gene products are thus properly expressed on the surface of B cells, which apparently interact normally with other cells of the mouse immune system. The mice generated a diverse antibody repertoire [8], reminiscent of adult human B cells. With the use of even larger DNA fragments from the human Ig loci, it should eventually be possible to recapitulate human antibody responses in mice. Such mice could be exploited to elucidate the molecular mechanisms underlying the programmed assembly and expression of human antibody genes during different stages of development, in both normal and abnormal situations, such as autoimmune diseases and other disorders.

The results I have described establish the introduction of megabase-sized human loci into the mouse germline as a powerful approach to elucidating the function and regulation of very large or crudely mapped genes. It can be used to study complementation of recessive genetic disorders, to generate dominant mutations that may provide models of human diseases, such as those involving partial chromosome trisomy, as well as to identify distant *cis*-acting elements involved in the control of gene expression, or in regulatory processes such as genetic imprinting and X-chromosome inactivation. Furthermore, a strategy such as the one I have described for the mouse antibody genes can be applied towards humanization of other multi-gene loci, such as the major histocompatibility complex or the T-cell receptor loci, that govern different compartments of the mouse immune system. Mice in which the endogenous loci are replaced by the analogous human genes will be useful in gaining insights into the structure–function relationships of the human loci and their involvement in the evolution of the immune system.

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Cloned transchromosomic calves producing human immunoglobulin

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Human polyclonal antibodies (hPABs) are useful therapeutics, but because they are available only from human donors, their supply and application is limited. To address this need, we prepared a human artificial chromosome (HAC) vector containing the entire unrearranged sequences of the human immunoglobulin (*hIg*) heavy-chain (*H*) and lambda (*λ*) light-chain loci. The HAC vector was introduced into bovine primary fetal fibroblasts using a microcell-mediated chromosome transfer (MMCT) approach. Primary selection was carried out, and the cells were used to produce cloned bovine fetuses. Secondary selection was done on the regenerated fetal cell lines, which were then used to produce four healthy transchromosomic (Tc) calves. The HAC was retained at a high rate (78–100% of cells) in calves and the *hIg* loci underwent rearrangement and expressed diversified transcripts. Human immunoglobulin proteins were detected in the blood of newborn calves. The production of Tc calves is an important step in the development of a system for producing therapeutic hPABs.

Despite the substantial need for hPABs to treat many diseases, the supply is limited to what can be obtained from human donors. Furthermore, the application of hPABs has been restricted because human donors cannot be hyperimmunized, that is, repeatedly boosted with antigen. Transgenic animals carrying *hIg* loci could provide a source of hPABs, especially targeted hPABs resulting from hyperimmunization with human pathogens or human molecules. Transgenic mice carrying *hIg* loci have been created[1–6] and are useful for the derivation of human monoclonal antibody therapeutics[7,8].

Transgenic cattle carrying *hIg* loci could be useful for large-volume commercial production of hPABs. Transgenesis including gene targeting[9–12] in livestock has been reported; however, the procedures used are not suitable for transfer of the *hIg* loci[13] (1–1.5 Mb for each locus) because the maximum size of DNA that can be inserted is very limited (20–100 kb). Mammalian artificial chromosome (MAC) vectors[14–17] may be a better choice because of their large insert capacity. Thus far, there have been no reports of the transfer of MAC vectors in livestock. Furthermore, human microchromosomes are generally mitotically unstable in a foreign environment[1,16,18]. This could be a major obstacle in the production of transchromosomic cattle, which require a large number of cell divisions for full term development.

Another potential limitation of using cattle to produce hPABs is the difference in immunophysiology between cattle and humans[19–22]. In humans and mice, bone marrow is the major site of origin of all lymphocytes and the location of subsequent B-cell maturation. In contrast, spleen, rather than bone marrow, is the presumed site of B-cell origin and immunoglobulin rearrangement in bovine. Furthermore, because of a limited number of functional V genes in bovine, gene conversion may be an important mechanism for the generation of diversity, especially for the light chain[19,20]. Gene conversion occurs in the Ileal Peyer's patch, where B cells undergo proliferation and diversification. These differences could impede the functional rearrangement, diversification, and production of hIgs in cattle.

In this study, we developed a system for introducing heavy- and light-chain *hIg* loci into bovine by transferring a 10 Mb HAC vector carrying the loci into primary fibroblast cells and then producing cloned cattle from the Tc cells. We also evaluated the retention of the HAC through early gestation and the functional rearrangement, diversification, and expression of hIgs in the blood of Tc calves.

Results

HAC transfer into bovine fetal fibroblasts and nuclear transfer. We constructed two HAC vectors (ΔHAC and ΔΔHAC), each carrying both *hIg* heavy-chain and λ light-chain loci, using a chromosome-cloning system[17,23] (Fig. 1). HAC vectors were introduced into bovine primary fetal fibroblasts from CHO clones using an MMCT system (Fig. 2). The life-span limits of bovine primary fibroblast cells required that complete antibiotic selection and DNA-based screening be avoided after MMCT to minimize cell divisions before nuclear transfer. Instead, we picked colonies on the basis of growth and morphology under selection and used them for nuclear transfer as quickly as possible. Nevertheless, the cells were useful only for a few days and could not be cryopreserved. Final selection was done after the rejuvenation and expansion of the cells during the growth of cloned fetuses. At 56–58 days, four

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Table 1. Development of cloned embryos derived from Tc fibroblasts

HAC	Nuclear transfer	Blastocysts (%)[a]	Blastocyst transfer	Recipients	Pregnant 40 days (%)	Pregnant 120 days (%)	Offspring (%)[b]
Δ	776	83 (18)	54	32	16 (50)	Fetal recovery	0
ΔΔ	833	122 (21)	56	28	13 (46)	Fetal recovery	1
Δ Regenerate	672	82 (17)	61	37	8 (22)	6 (16)	6 (16)

Fetal recovery was done between 56 and 119 days of gestation. Regenerated cells were produced from Tc fetuses recovered at 56–58 days of gestation.
[a]Percentage of blastocysts was calculated as the number of blastocysts per number of fused donor cell oocyte complexes. Average fusion rate in our laboratory was 70%.
[b]For ΔΔHAC nuclear transfers, one fetus was not recovered and developed to term producing a five healthy calf. For ΔHAC regenerated cells, six live calves were born; two from cell line 6032 did not survive past 48 h and the other four remain alive and healthy.

ΔHAC and two ΔΔHAC fetuses were recovered and fibroblast cell lines were regenerated, expanded, and cryopreserved for further analysis and nuclear transfer. Efficiency of development to the blastocyst stage, to 40 days of gestation, and to term is shown in Table 1 for first-generation and recloned cells.

Analysis of cloned Tc fetuses. To examine whether the HAC vector was retained through early gestation and whether h*Ig* loci could be functionally rearranged and expressed during early B-cell development in bovine, we analyzed cloned Tc fetuses collected between 56 and 119 days of gestation. Retention of the HACs in the fibroblast lines derived from six cloned Tc fetuses collected at 56–58 days and from an additional seven fetuses collected between 77 and 119 days was evaluated by G418 resistance (Fig. 3A) and by genomic PCR of human *IgH* and h*Igλ* loci (Fig. 3B). Of the 13 fetuses, 9 were resistant to G418 (4 ΔHAC and 5 ΔΔHAC) and 8 showed the presence of both human *IgH* and *Igλ* loci. We evaluated five positive 77–119-day fetuses for expression and rearrangement of the h*Ig* loci by reverse transcription–PCR (RT-PCR) analysis, followed by sequencing of the amplified products. Human *IgH* and *Igλ* genes were expressed (Fig. 3C) in all fetuses, predominantly in spleen, consistent with endogenous bovine *Ig* expression (data not shown). The sequences showed evidence of functional V(D)J recombination (Fig. 3D). These results demonstrate retention of the HAC vector, functional V(D)J recombination, and expression of the h*Ig* locus.

Generation of cloned Tc calves. For production of cloned Tc calves, the three regenerated ΔHAC cell lines (5968, 6032, and 6045) were used for recloning. One male calf from cell line 6045 and five female calves from cell lines 5968 and 6032 were produced from 37 recipients (16%, Fig. 4A). Four calves survived and were healthy and phenotypically normal. Retention of the HAC vector was confirmed in all the calves by G418 selection (data not shown), genomic PCR (Fig. 4B), and fluorescent *in situ* hybridization (FISH) analyses (Table 2; Fig. 4C). FISH analysis indicated that the HAC was retained as an independent chromosome and that the proportion of cells retaining the HAC ranged from 78% to 100%. We observed no obvious differences in retention rates between peripheral blood lymphocytes (PBLs; 91%) and fibroblasts (87%). These data demonstrate that somatic-cell recloning strategies can be used to produce healthy cloned Tc calves and that the HAC vector can be stably maintained through the large number of cell divisions in bovine development.

Human *Ig* gene expression and protein production in Tc calves. To determine whether h*Ig* loci were rearranged and expressed, we carried out RT-PCR analysis on PBLs. We observed expression of both human *IgH* and *Igλ* genes in the PBLs of all the calves (data not shown). The diversity of the human *IgH* and *Igλ* repertoire was determined by sequence analysis (Table 3). A representative set of the sequences showed a wide utilization of VH/Vλ, D, and JH/Jλ segments distributed over the loci. In the *IgH* transcripts, the frequent utilization of JH4 and of V segments from VH1 and VH3 was observed, similar to patterns in human[24]. Addition of non-germline nucleotides (N addition) and nucleotide deletion were also observed in both hIgH and hIgλ transcripts. These produced a high degree of diversification in the third complementarity-determining region of both the h*IgH* and h*Igλ* chains. Furthermore, hIg proteins were secreted at levels ranging from 13 ng/ml to 258 ng/ml (immunoglobulin expression is typically very low to undetectable in newborn calves[25]) in blood samples collected before colostrum feeding in five of the seven Tc calves. In the two calves in which hIg proteins were not detected, bovine cell lines (5968, 6032, and 6045) were used for recloning. One male calf from cell line 6045 and five female calves from cell lines 5968 and 6032 were produced from 37 recipients (16%, Fig. 4A). Four calves survived and were healthy and phenotypically normal. Retention of the HAC vector was confirmed in all the calves by G418 selection (data not shown), genomic PCR (Fig. 4B), and fluorescent *in situ* hybridization (FISH) analyses (Table 2; Fig. 4C). FISH analysis indicated that the HAC was retained as an independent chromosome and that the proportion of cells retaining the HAC ranged from 78% to 100%. We observed no obvious differences in retention rates between peripheral blood lymphocytes (PBLs; 91%) and fibroblasts (87%). These data demonstrate that somatic-cell recloning strategies can be used to produce healthy cloned Tc calves and that the HAC vector can be stably maintained through the large number of cell divisions in bovine development.

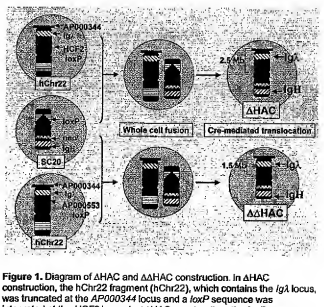

Figure 1. Diagram of ΔHAC and ΔΔHAC construction. In ΔHAC construction, the hChr22 fragment (hChr22), which contains the *Igλ* locus, was truncated at the *APB00344* locus and a *loxP* sequence was integrated at the *HCF2* locus. In ΔΔHAC construction, the *loxP* sequence on the hChr22 fragment was integrated at the *APB00553* locus. DT40 cells containing the hChr22 fragments were fused with DT40 cells containing the SC20 fragment (SC20 is a fragment of hChr14 and contains the *IgH* locus). Cre-mediated translocation resulted in ΔHAC, which contained a 2.5 Mb hChr22 region, and ΔΔHAC, which contained a 1.5 Mb hChr22 region, each fused to the SC20 vector.

Table 2. HAC retention in cloned Tc calves

Calf number	Cell type	HAC positive/ total (%)	Two signals/ total (%)
50	PBL	50/50 (100)	6/50 (12)
50	Fibroblast	47/50 (94)	0/50 (0)
1064	PBL	46/50 (92)	0/50 (0)
1064	Fibroblast	34/39 (87)	3/39 (8)
1065	PBL	39/50 (78)	2/50 (4)
1065	Fibroblast	49/60 (82)	0/60 (0)
1066	PBL	47/50 (94)	1/50 (2)
1066	Fibroblast	43/50 (86)	0/50 (0)
Total	Combined	355/399 (89)	12/399 (3)

Retention rate was determined in both PBLs and fibroblasts in each calf by FISH analysis using human Cot-1 DNA as a probe.

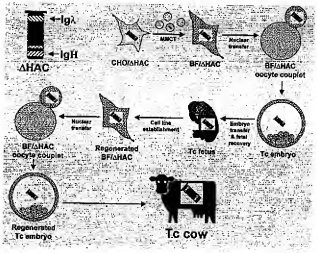

Figure 2. Procedure for production of cloned Tc calves. Structure of ΔHAC (upper left) is shown with hChr22 (green) and hChr14 (red) regions containing *Igλ* and *IgH* loci. The centromere of the HAC is derived from the hChr14 fragment. The HAC was transferred from a CHO clone (CHO/ΔHAC, light blue) into fetal bovine fibroblasts by means of a MMCT technique. Tc fibroblasts (BF/ΔHAC, black patterned) and enucleated oocyte (yellow) couplets were fused, resulting in transfer of the fibroblast nucleus and formation of an embryo. The reconstituted Tc embryos were cultured *in vitro* to the blastocyst stage and then implanted into recipient cows. At ~60 days of gestation, Tc fetuses were recovered and Tc fibroblast cell lines were re-established (regenerated BF/ΔHAC), evaluated, and used for further nuclear transfer. Regenerated Tc embryos were transferred to recipients to produce Tc calves.

immunoglobulin proteins were also not detected. These results demonstrate that the human *IgH* and *Igλ* loci can be functionally rearranged and expressed in cattle.

Discussion

In this study, we demonstrate the production of healthy calves carrying a stable microchromosomal vector encoding human *IgH* and *Igλ* loci, functional diversification of the h*Ig* genes, and production of hIg protein. To accomplish this, several challenges needed to be addressed, including the lack of methods for introducing large foreign DNA sequences into cattle, the possible mitotic instability of a microchromosome vector, and the substantial differences in immune system development between cattle and humans.

MMCT has been accomplished in mice using embryonic stem cells and chimera production followed by germline transmission from embryonic stem cell–derived germ cells[1]. Mouse embryonic stem cells have the advantage of an unlimited life span, rapid growth in culture, a straightforward system for clonal propagation of transgenic colonies, and a high rate of contribution to the germ line in chimeras. Unfortunately, embryonic stem cells with similar properties have not been derived in other species, including cattle[26]. It was therefore necessary to use a nuclear transfer system with primary somatic cells[27] to produce Tc cattle. The limitation of this system is that primary fibroblasts, the cell type of choice, have a life span of ~35 population doublings[28]. After MMCT, our cells were capable of dividing for only about one week and could not be cryopreserved. Our approach to solving this problem was to carry out incomplete selection after MMCT and complete selection on rejuvenated cloned cell lines, and then produce calves by recloning cell lines. Recloning of transgenic somatic cells and production of offspring has been accomplished in cattle[29] with limited success. Our results indicate that rejuvenation of cell lines by recloning is a viable method for the production of large numbers of transgenic animals and the establishment of cryopreserved transgenic cell banks.

In mice, the SC20 HAC vector is retained in 70–80% of fibroblasts[1] and only 30–40% of PBLs (data not shown), revealing limitations in mitotic stability in a foreign environment. Because of the number of cell divisions required for bovine development, the retention rate of the HAC was expected to be low. However, the HAC was retained in all cloned calves at a very high rate, both in

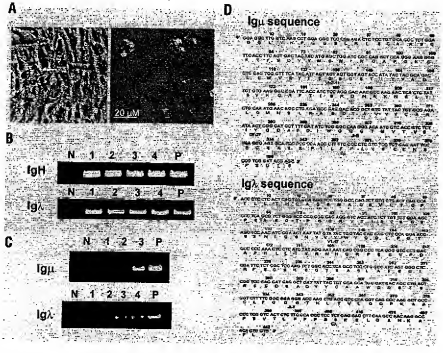

Figure 3. Analysis of Tc fetuses. (A) G418 selection of regenerated Tc fibroblast line (left) and control nontransgenic fibroblasts (right). (B) Genomic PCR of *IgH* and *Igλ* loci in Tc fetuses and controls. The three fetuses, 5968 (lane 1), 6032 (lane 2), and 6045 (lane 3) were derived from ΔHAC fibroblasts; fetus 5580 (lane 4) was derived from ΔΔHAC fibroblasts. As a control, a nontransgenic fetus (lane N) was recovered and evaluated. Human *IgH* and *Igλ* loci were detected by PCR in all Tc fetuses and in a positive-control human liver DNA sample (lane P), but not in the negative control (lane N). (C) Rearranged and expressed human Igμ and Igλ transcripts amplified by RT-PCR from negative-control nontransgenic bovine spleen (lane N), from brain (lane 1), liver (lane 2), and spleen (lane 3 and 4) of cloned Tc fetus, and from positive-control human spleen (lane P). (D) A representative nucleotide and deduced amino acid sequence of human Igμ and Igλ transcripts amplified by RT-PCR from a cloned Tc fetus recovered at 99 days. In the Igμ transcript, blue represents the variable region sequence (Vh3-11), red represents the diversity region (D7-27), green represents the joining region (Jh3) and orange represents the constant region. In the Igλ transcript, blue represents the variable region sequence (Vλ1-17), red represents the joining region (Jλ3), and green the constant region sequence.

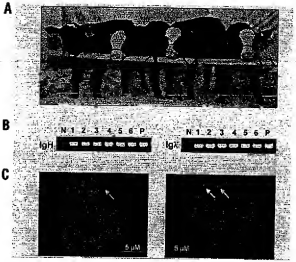

Figure 4. Analysis of cloned Tc calves. (A) Four cloned Tc calves: male calf (60) from cell line 6045 and female calves (1064, 1065, 1068) from cell line 5968. (B) Genomic PCR of *IgH* and *Igλ* loci in PBLs from cloned Tc calves and controls: calf 1064 (lane 1), 1065 (lane 2), 1066 (lane 3), 50 (lane 4), 1067 (lane 5), and 1068 (lane 6). Both human *IgH* and *Igλ* loci were detected by genomic PCR in all the Tc calves and positive-control human liver DNA (lane P), but not in a negative-control nontransgenic calf (lane N). (C) FISH analysis in metaphase chromosome spreads in a cell showing a single signal and a cell showing a double signal. Arrows indicate location of HACs (red) among surrounding bovine chromosomes (blue). A single HAC per cell is introduced and retained in most cells (left panel); however, improper separation of chromatids at cell division may result in some cells having two microchromosomes (right panel) and some not having a microchromosome.

fibroblasts (87%) and PBLs (91%). In our previous study[17], the mitotically unstable hChr22 fragment was mitotically stabilized when its centromere was exchanged with that of the SC20 vector, suggesting that the mitotic stability of human microchromosomes might be affected by centromeric structure. In this study, the unexpectedly high mitotic stability of the HAC vector in bovine may be explained by greater compatibility of the human centromeric structure with factors regulating cell division in cattle as compared with mice. The high rate of HAC retention shows the utility of HAC vectors for stable introduction of foreign genetic elements into the genome of cattle.

We have shown that h*Ig* loci can be functionally rearranged and expressed with substantial diversity in cattle despite the differences in immunophysiology between cattle and humans. Both broad usage of VDJ segments and N addition contributed considerably to h*Ig* diversity in human IgH and Igλ transcripts in cloned Tc calves. Notably, in bovine Igλ transcripts, N addition is rarely observed (data not shown). These results indicate that immunoglobulin gene diversification may be more related to genetic sequence than to the environment in which diversification occurs.

Table 3. Repertoire analysis of human immunoglobulin heavy- and λ-chain transcripts in cloned Tc calves

Human μ nucleotide sequences

VH	N	DH	N	JH
6-1	0	D5-24	3	JH3
TACTGTGCA		AGAGATG	AGA	ATGCTTTTGATGTC
3-33	8	D6-13	3	JH4
ATTACTGTGCGA	AGAACAAA	ATAGCAGCAGCTGGTAC	GAT	CTTTGACTACT
3-15	4	D6-19	4	JH1
ACTGTACCACAGA	TCTG	ATAGCAGTGGCTGGTAC	TGGG	TACTTCCAGCA
3-66	2	D2-2	0	JH3
TACTGTGCGAG	TC	GTAGTACCAGCTGCTAT		GATGCTTTTGATGTCT
3-21	6	D2-21	8	JH4
TTACTGTGCGAG	TTTTGG	GTGGTGGT	CACATTTA	GACTACTGGGG
4-39	8	D3-10	3	JH4
ACTGTGCGAGACA	TGAAAAAC	TTCGGGGAGTTAT	AAT	CTACTGGGCCC
1-69	7	D6-13	1	JH4
TTACTGTGCGAG	GGGGATG	GCAGCAGCTGGTAC	C	GACTACTGGGGC
1-8	0	D2-2	12	JH2
ACTGTGCGAGAG		ATTGTAGTAGTACCAGCTGC	CAAGATCGTAAG	TGGTACTTCGAT
1-18	0	D5-24	15	JH4
TTACTGTGC		GAGATGG	GTTTTTGATCCCCAG	TTTGACTACTGG
3-20	4	D7-27	1	JH3
TCACTGTGCGAGAA	TTTT	ACTGGGGA	T	GATGCTTTTGATGTCT

Human λ nucleotide sequences

Vλ	N	Jλ
1-17 AGCCTGAGTGGTC	2 (TT)	Jλ3 TTCGGCGGAGGG
2-13 CAGTGGTAACCATCT	0	Jλ2 GGTATTCGGCGGAGG
1-19 CAGCCTGAGTGCTG	0	Jλ1 TCTTCGGAACTGGG
5-2 AGCAACTTCGTGTA	2 (TA)	Jλ3 GTTCGGCGGAGAG
1-7 GGTAGTAGCACTT	1 (C)	Jλ3 TCGGCGGAGGGA
2-13 CAGTGGTAACCAT	0	Jλ1 TATGTCTTCGGAACTG
2-1 GACAGCAGCACT	0	Jλ1 TATGTCTTCGGAACTG
1-2 GGCAGCAACAATTTC	1 (G)	Jλ1 ATGTCTTCGGAACTG
1-4 AGCAGCAGCACTC	2 (GT)	Jλ3 TTCGGCGGAGG
1-4 AGCAGCAGCACTC	0	Jλ1 GGAACTGGGA

Human μ- and λ-specific mRNAs were amplified by RT-PCR, cloned, and sequenced. Nucleotide sequences of VDJ junctions of each of ten independent μ and λ clones are shown, divided into VH/Vλ, D, JH/Jλ, and N segments, as identified by homology to published germline sequences (Ig-BLAST).

Several challenges remain before our system can be used for large-scale production of hPABs. Because Tc cattle retain the bovine *Ig* loci, expression of bovine antibody is expected to dominate over that of human antibody. We have observed this previously in Tc mice in which the murine *Ig* loci were not inactivated (data not shown). Furthermore, chimeric antibodies containing combinations of human and bovine heavy- and light-chain proteins are expected to be present. Therefore, methods of reducing bovine *Ig* expression are probably needed before commercial production of hPABs can proceed in Tc cattle.

A Tc bovine–based system for producing therapeutic hPABs would have several advantages: (i) cattle could be hyperimmunized with essentially any human pathogen or human molecule; (ii) cattle produce very large quantities of antibodies; (iii) large numbers of antigens could be evaluated quickly because one line of genetically modified cattle could be used for all antigens; and (iv) scale-up of antibody production would be as straightforward as immunizing additional cows. Therapeutic hPABs produced in a Tc bovine–based system may have broad application in the treatment and prevention of infectious disease (including antibiotic resistant infections), autoimmune disease, and cancer.

Experimental protocol

Construction of HAC vectors. HACs were constructed using a previously described chromosome-cloning system[17,23] (Fig 1). Briefly, for the construction of ΔHAC, the previously reported hChr22 fragment (hChr22) containing a *loxP* sequence integrated at the *HCF2* locus was truncated at the *AP000344* locus by telomere-directed chromosomal truncation. Next, cell hybrids were formed by fusing the DT40 cell clone containing the hCF22 with a DT40 cell clone containing the stable and germline-transmittable human microchromosome vector SC20. The resulting DT40 cell hybrids contained both hChr fragments. The DT40 hybrids were transfected with a Cre recombinase expression vector to induce Cre–*loxP*-mediated chromosomal translocation between hChr22 and the SC20 vector. The stable transfectants were analyzed using nested PCR to confirm the cloning of the 2.5 Mb hChr22 region into the *loxP*-cloning site in the SC20 vector (ΔHAC). ΔΔHAC was constructed using the same chromosome cloning system except that the *loxP* sequence on hChr22 was integrated into the *AP000553* locus, creating a 1.5 Mb insert upon Cre–*loxP*-mediated translocation.

HAC vector transfer into bovine fetal fibroblasts. Bovine fetal fibroblasts were cultured in α-MEM (Life Technologies, Rockville, MD) medium supplemented with 10% (vol/vol) FCS (Life Technologies) at 37°C and 5% CO_2. Microcells were purified from the CHO clone retaining the ΔHAC or ΔΔHAC as described previously[17]. Bovine fetal fibroblasts were fused with microcells using polyethylene glycol (PEG 1500, Roche, Nutley, NJ), and the fused cells were selected under 700 μg/ml of G418 (Life Technologies) for 10–14 days. The G418-resistant clones were picked and used for nuclear transfer.

Nuclear transfer. The nuclear transfer procedure was carried out essentially as described previously[27,28]. *In vitro*–matured oocytes were enucleated ~18–20 h post maturation. Cytoplast-donor cell couplets were fused using a single electrical pulse of 2.4 kV/cm for 20 μs (Electrocell Manipulator 200, Genetronics, San Diego, CA). At 30 h, post meturation reconstructed oocytes were activated with calcium ionophore (5 μM) for 4 min (Cal Biochem, San Diego, CA) and 10 μg cycloheximide and 2.5 μg cytochalasin D (Sigma) as described earlier[28]. After activation, cloned embryos were placed in culture in four-well tissue culture plates, containing irradiated mouse fetal fibroblasts and 0.5 ml of ACM culture medium covered with mineral oil (Sigma) and incubated at 38.5°C in a 5% CO_2 in air atmosphere. On day 4, 10% (vol/vol) FCS (Life Technologies) was added to the culture medium. On days 7 and 8, embryos were transferred into synchronized recipients. All animal work was done following a protocol approved by the Trans Ova Genetics (Sioux Center, IA) institutional animal care and use committee.

Genomic PCR analysis. Genomic DNA was extracted from Tc fetuses at 56–119 days of gestation or from cloned newborn calves and subjected to PCR using primers IGHV3 for the human *IgH* locus and IGLC for the human *Igλ* locus, as described previously[17].

RT-PCR and repertoire analyses. Total RNA was recovered from spleen, liver, and brain of Tc fetuses or from PBLs of Tc calves. RT-PCR was carried out as described previously[30]. For human Igμ transcripts, VH1/5 BACK, VH3 BACK, and VH4BACK were used as a 5′ primer and Cμ-2 was used as a 3′ primer. For human Igλ transcripts, Vλ1LEA1, Vλ2MIX, and Vλ3MIX were used as a 5′ primer and CλMIX was used as a 3′ primer. The amplified cDNAs were subcloned by using a TA cloning kit (Invitrogen, San Diego, CA) and sequenced using e DNA autosequencer (ABI3700 Sequencer, GlaxoWellcome, Herts, United Kingdom).

FISH enelysis. Digital image analysis was done using the Mac Probe system (Applied Imaging, Santa Clara, CA). HAC painting was done using digoxigenin-labeled (Boehringer Ingleheim, Ridgefield, CT) human Cot-1 DNA as a probe, and the digoxigenin signal was detected with an anti-digoxigenin–rhodamine complex that fluoresced red. DAPI (Sigma) was used for background staining. Standard chromosome and FISH protocols were carried out as described[31].

ELISA analysis for human antibody. Plasma samples were obtained from Tc calves before they were fed colostrum end human Ig levels were determined by solid-phase ELISA. The assay used a bovine anti-human immunoglobulin as the capture antibody and an HRP-labeled sheep anti-human immunoglobulin as the detecting antibody. Amounts of human immunoglobulin >10 ng/ml were reliably detected by this assay.

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Competing interests statement
The authors declare competing financial interests: see the Nature Biotechnology website (http://www.nature.com/naturebiotechnology) for details.

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A Tissue-specific Transcription Enhancer Element Is Located in the Major Intron of a Rearranged Immunoglobulin Heavy Chain Gene

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Summary

We have studied the DNA sequences required for high level expression of a cloned heavy chain immunoglobulin gene stably introduced into mouse myeloma cells by DNA transfection. We found that DNA sequences derived from the germ line J_H–C_γ region are required for accurate and efficient transcription from a functionally rearranged V_H promoter. Similar to viral transcriptional enhancer elements, these cellular sequences stimulate transcription from either the homologous V_H gene segment promoter or a heterologous SV40 promoter. They are active when placed on the 5′ or 3′ side of the rearranged V_H gene segment and they function when their orientation is reversed. However, unlike viral enhancers, the Ig gene enhancer appears to act in a tissue-specific manner, since it is active in mouse B cells but not in mouse fibroblasts. The nucleotide sequence of the Ig enhancer region contains repeating elements that closely resemble sequence elements found in many viral enhancers. We discuss the possible role of tissue-specific transcription in cell differentiation and malignant transformation.

Introduction

Molecular analyses of immunoglobulin (Ig) genes established that an Ig polypeptide chain is encoded in multiple gene segments scattered along a chromosome of the germ line genome and that these gene segments must be brought together to form a complete Ig gene active in B lymphocytes (For a review see Tonegawa, 1983). This somatic assembly of Ig gene segments is achieved by a series of developmentally-controlled recombination events that occur during the differentiation of B cells.

Two types of recombination occur: V–J or V–D–J joining and switch recombination. The V–J and V–D–J joinings are essential for the formation of the DNA sequence coding for the variable (V) region of the light and heavy chain, respectively, while switch recombination replaces the constant (C) region-coding sequence of the heavy chain of one class (usually μ) with another (γ, ϵ, α, etc.). The exact timing of these recombination events in the course of B cell development has not been determined, but both V–J and V–D–J joining occur before the cell encounters antigens.

One of the most important functions of the V–J and V–D–J joinings is to create complete Ig genes with a diverse set of V region-coding DNA sequences from a limited number of the gene segments carried in the germ line genome (Tonegawa, 1983). Another important function of V–J and V–D–J joining events is in the control of the expression of Ig genes during B cell differentiation. On the basis of the fine structural analysis of Ig genes, it is clear that these joining events are prerequisite for the synthesis of a complete Ig chain: there is no evidence that any unrearranged germ line Ig gene segment can directly participate with its coding capacity in the synthesis of a complete, functional Ig chain (Brack et al., 1978; Bernard et al., 1978; Sakano et al., 1979; Max et al., 1979; Early et al., 1980; Sakano et al., 1980). In fact, it has been shown that in a myeloma cell the RNA transcript of an unrearranged V, segment is no more than 0.1 copy per gene, a level at least four orders of magnitude lower than that of the transcript from the rearranged, expressed V, segment present in the same cell (Mather and Perry, 1981).

Both the V–J and V–D–J joining events alter the sequence configurations in the 3′ region of the germ line V gene segment, but the 5′ flanking region of the V gene segment, where the transcription promoter and other controlling elements reside, is unaffected by the rearrangement (Bernard et al., 1978 and Clarke et al., 1982). Although a few nucleotide differences have been found between the germ line and somatic sequences in the 5′ flanking region of V gene segments (Sakano et al., 1980), these base substitutions are by-products of the somatic mutation events whose physiological role is to diversify the V-coding sequences (Bernard et al., 1978; Weigert and Riblet, 1976; Selsing and Storb, 1981). These base changes are not systematic and therefore are thought to have no bearing on the control of Ig gene expression (Clarke et al., 1982).

A possible explanation of how the downstream sequence might confer transcriptional competence to the rearranged V gene segment promoter is through transcriptional enhancement. Although the mechanism of this phenomenon is unknown, specific viral DNA sequence elements have been described (Banerji et al., 1981; de Villiers et al., 1981; Levinson et al., 1982) which enhance viral or recombinant cellular gene transcription. Because such enhancer elements can activate transcription from promoters which are located either upstream or downstream, and more than 1 kb away, it is possible that an analogous element might be located near the C gene segment. The observations that the C gene segments, in contrast to the V gene segments, are transcriptionally active in lymphoid cells in the absence of rearrangement support this hypothesis. In this case, promoter-like sequences upstream of the C gene segments are utilized for transcription but the

transcripts are degraded in the nuclei (Kemp et al., 1980; Van Ness et al., 1982).

The recent technical advances for introducing cloned immunoglobulin genes into lymphoid cells (Oi et al., 1983; Rice et al., 1983) have made it possible to study the structure–function relationship between specific DNA sequences and gene expression in these cells. We describe an enhancer element in the major intron of a rearranged γ_{2b} heavy chain gene. This sequence is located between the J_H region and the switch-recombination site utilized in myeloma MOPC 141, i.e., it is derived from sequences upstream of the germ line C_γ gene segment.

Results

High Level Expression of the Heavy Chain Gene Introduced into Myeloma Cells

We previously reported that the functionally rearranged immunoglobulin heavy chain (γ_{2b}) gene from MOPC 141 tumor cells (Sakano et al., 1980) can be accurately expressed at a low level in transfected mouse L cells (Gillies et al., 1983). For the studies presented here, we subcloned the same γ_{2b} gene fragment into plasmid pSV2*gpt* (Mulligan and Berg, 1980), transfected the mouse myeloma line, J558L, and selected for *gpt* gene activity by resistance to mycophenolic acid. This J558L line has lost the ability to express the endogenous immunoglobulin heavy chain gene but continues to synthesize a λ light chain. Furthermore, J558L has been shown to have a relatively high transformation frequency ($>10^{-4}$) when pSV2*gpt* vectors containing light chain genes are used for transfection (Oi et al., 1983).

Using a modified protocol for protoplast fusion (see Experimental Procedures), we found that plasmid pSV-γ_{2b}VC (Figure 1) transforms J558L cells at a frequency of greater than 10^{-3}. This high frequency made it possible to use pools of independently-derived clones of *gpt* transformants to compare the expression of plasmids containing defined deletions with that of the parental plasmid pSV-γ_{2b}VC. The advantage of this method is that the resulting cell lines represent several independent integration events (required for transformation), therefore the level of heavy chain gene expression in a given pool should reflect the average level of the individual clones. Thus the possible effect of the site of integration on the expression of the transfected gene is minimized.

Cell lines obtained by transfection with plasmid pSV-γ_{2b}VC and selection for *gpt* expression (growth in the presence of mycophenolic acid) were found to express high levels of γ_{2b} heavy chain (Figure 2A, lanes 2–5). These levels of expression of the exogenous γ_{2b} genes are estimated to be about 20% of that of the endogenous γ_{2b} gene in MOPC 141. Apparently, this heavy chain can form an immunoglobulin molecule with the λ light chain of myeloma J558L, because the light chain was immunoprecipitated from cell extracts with antiheavy chain antisera and equimolar amounts of heavy and light chain were secreted into the culture medium (Figure 2A, lane 14).

A Deletion of Part of the Major Intron Abolishes the High Level Expression of the Heavy Chain Gene

Deletion mutants of the parental plasmid were constructed to test whether the removal of specific noncoding DNA sequences would affect the expression of the γ_{2b} gene in J558L cells. Because deletions between the VDJ and C_μ exons of an Abelson murine leukemia virus-transformed cell line have been correlated with decreased heavy chain production (Alt et al., 1982), we constructed mutant plasmids with deletions in this region. Two such plasmids, pSV-γ_{2b}3′RΔ1 and pSV-γ_{2b}3′RΔ2 contain overlapping deletions around the unique Eco RI site of the parental plasmid pSV-γ_{2b}VC (Figure 1). These three plasmids were introduced into J558L myeloma cells and the expression of the γ_{2b} heavy chain gene in stably transformed cells was compared.

Cell lines obtained by transfection with plasmid pSV-γ_{2b}3′RΔ1 synthesized high levels (no less than half of the wild type level) of γ_{2b} heavy chain (Figure 2A, lanes 6–9) and secreted immunoglobulin (Figure 2A, lane 15). In contrast, four cell lines obtained by transfection with plasmid pSV-γ_{2b}3′RΔ2 synthesized only low levels (about 5% of the wild type level) of heavy chain (Figure 2A, lanes 10–13). The same results were obtained when subclones of

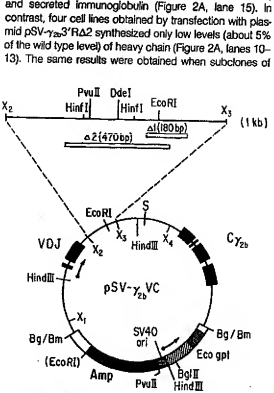

Figure 1. Partial Restriction Map of Plasmid pSV-γ_{2b}VC

A 9 kb Bgl II fragment was inserted into the Bam HI site (indicated by Bg/Bm) of plasmid pSV2*gpt*. The Ecogpt gene (wide stripes) is flanked by SV40 sequences (thin stripes) including the origin (ori) of replication and mRNA start site (arrow). The γ_{2b} gene (narrow line) contains VDJ and $C_{\gamma 2b}$ exons (solid boxes) and a mRNA start site (arrow) about 30 bp upstream of the VDJ coding sequence (Gillies and Tonegawa, 1983). The switch recombination (S) site is also shown. The DNA segments deleted in plasmids pSV-γ_{2b}3′RΔ1 and pSV-γ_{2b}3′RΔ2 are shown in linear form above the circular map. The sizes of the deletions, as determined by restriction analysis, are indicated. The exact locations of these deletions are shown in Figure 7.

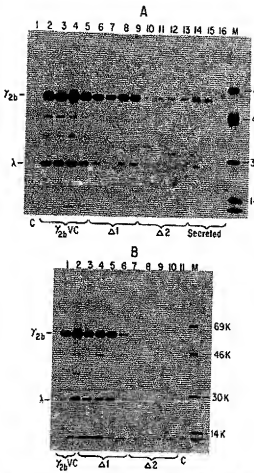

Figure 2. Expression of γ_{2b} Heavy Chain Protein in Transfected Cells

Transfected cell lines were labeled with ^{35}S-methionine and cell extracts were analyzed as described in Experimental Procedures. Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. (A) Four transfected lines (pools of individual clones) were analyzed for each plasmid tested. Lane 1: control J558L cells; lanes 2–5: cells transfected with plasmid pSV-γ_{2b}VC; lanes 6–9: cells transfected with plasmid pSV-γ_{2b}3′RΔ1; lanes 10–13: cells transfected with plasmid pSV-γ_{2b}3′RΔ2. Secreted proteins from cells transfected with plasmid pSV-γ_{2b}VC (lane 14), plasmid pSV-γ_{2b}3′RΔ1 (lane 15), and plasmid pSV-γ_{2b}3′RΔ2 (lane 16) were immunoprecipitated and analyzed on the same gel. (B) Cell lines subcloned from the transfected cell lines were tested for γ_{2b} heavy chain protein synthesis as in (A). The plasmids used for transfection are indicated below the autoradiograms. Control (C) cell extract is shown in lane 11. The positions of the γ_{2b} heavy chain and λ light chain (synthesized in J558L cells but not immunoprecipitated in the absence of γ_{2b} heavy chain) are indicated.

each pool were tested for γ_{2b} heavy chain expression (Figure 2B), although more variation was observed in the level of expression between individual clones. Nonetheless, these results strongly suggest that DNA sequences deleted in plasmid pSV-γ_{2b}3′RΔ2, but still present in pSV-γ_{2b}3′RΔ1, are essential for the high level expression of heavy chain genes in myeloma cells.

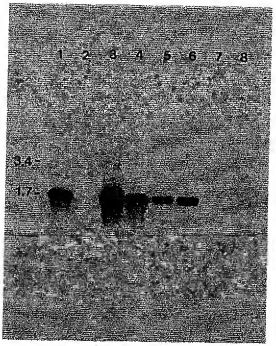

Figure 3. Northern Gel Blotting Analysis of Transfected Cell RNA

Total cell RNA (10 μg per lane) was electrophoresed on a denaturing agarose gel, transferred to nitrocellulose, and hybridized to nick-translated Cγ_{2b} probe. RNA (2.5 μg) from MOPC 141 cells (lane 1) and J558L cells (lane 2) were also analyzed. Two cell lines transfected with plasmid pSV-γ_{2b}VC (lanes 3 and 4), plasmid pSV-γ_{2b}3′RΔ1 (lanes 5 and 6), and plasmid pSV-γ_{2b}3′RΔ2 (lanes 7 and 8) are shown. The positions of the secreted (1.7 kb) and membrane forms (3.4 kb) of γ_{2b} mRNA are indicated.

The Reduced Expression of the γ_{2b} Gene Is at the Level of RNA

The steady-state level of γ_{2b} mRNA in transfected cell lines was analyzed by Northern gel blotting and hybridization with the Cγ_{2b} probe. It should be noted that the γ_{2b} heavy chain gene used in these studies does not contain the exons coding for the membrane form of γ_{2b} (Gillies and Tonegawa, 1983) and thus the only species of mRNA expected in transfected cells is the secreted form (1.7 kb).

As seen in Figure 3, cell lines transfected with plasmid pSV-γ_{2b}VC (lanes 3 and 4) and plasmid pSV-γ_{2b}3′RΔ1 (lanes 5 and 6) contain high levels of the secreted form of γ_{2b} mRNA. The cell lines transfected with plasmid pSV-γ_{2b}3′RΔ2 (Figure 3, lanes 7 and 8) contained much lower levels of γ_{2b} mRNA of the correct size, in agreement with the decreased level of γ_{2b} heavy chain protein (Figure 2). The additional RNA bands seen in lanes 7 and 8 (Figure 3) also contain γ_{2b} sequences but appear to be read-through products of the Ecogpt gene. Data presented below support this explanation, as opposed to the idea that the intron deletion has a deleterious effect on RNA splicing and results in low levels of translatable γ_{2b} mRNA.

Plasmid Copy Number in Transfected Cell Lines

DNA from transfected cells was analyzed by Southern gel blotting to determine the plasmid copy number and its possible effect on the level of γ_{2b} mRNA. When a pSV2*gpt* plasmid DNA probe was used for hybridization, a striking difference in plasmid copy number was found (Figure 4). Two prominent bands, corresponding to the two large Hind III fragments (6.2 kb and 5.0 kb) common to all the plasmids, are detected with this probe (seen best in Figure 4, lanes 6 and 7). Clearly, the DNA sequences deleted in plasmid pSV2-γ_{2b}3′RΔ2 (those required for the high level expression of γ_{2b} mRNA) have a dramatic effect on the number of copies of plasmid required for transformation to the *gpt*$^+$ phenotype. When these sequences are present, as they are in plasmids pSV-γ_{2b}VC and pSV-γ_{2b}3′RΔ1, a low copy number is sufficient for *gpt* transformation (Figure 4, lanes 2–5). In the absence of these sequences, the copy number is increased at least 20-fold (Figure 4, lanes 6 and 7), presumably to compensate for a comparable decrease in *gpt* mRNA transcription.

Two conclusions can be made from these results: one, the DNA sequences required for the high level expression of γ_{2b} mRNA also increase the level of expression from the heterologous SV40 promoter at least 20 times; two, the level of RNA transcribed from the V gene segment promoter is decreased about 400 times per gene copy in the absence of this DNA sequence. This calculation is based on the observed decrease by a factor of 20 in γ_{2b} gene expression as a result of the 3′RΔ2 deletion, and the fact that this decreased level is likely the result of the transcription of at least 20 times as many gene copies.

DNA Sequences Located in the γ_{2b} Gene Intron Enhance Expression in an Orientation-independent and a Position-independent Manner

The DNA sequences defined as viral enhancer elements have been shown to stimulate the transcription of homologous or heterologous promoters either upstream or downstream, and in either orientation with respect to the direction of transcription (Moreau et al 1981; Wasylyk et al, 1983). In order to test whether the sequences located in the major intron of the γ_{2b} gene (and all other heavy chain genes) behave similarly, we constructed a plasmid with most of the intron sequences deleted. We then inserted a 1 kb Xba I fragment ($X_{2/3}$) containing those intron sequences with potential enhancer activity into either of two sites in either of the two orientations. The first corresponds to the original position of this fragment in the parental plasmid (as part of the VDJ-Cγ_{2b} intron) and the second is approximately 1.4 kb upstream (on the 5′ side of the V gene segment). Four plasmids were obtained which contained the $X_{2/3}$ fragment in the normal or reversed orientation, either upstream or downstream of the mRNA start site (see Figure 5A).

Cell lines obtained by transfection with the plasmids just described were analyzed for the expression of γ_{2b} heavy chain. As seen in Figure 5B, cells transfected with plasmid pSV-$\gamma_{2b}\Delta X_{2/4}$ (with most of the intron deleted) did not synthesize significant levels of γ_{2b} protein (lanes 3 and 4). The insertion of the $X_{2/3}$ fragment into the intron site (the normal position of this fragment) restored the expression of γ_{2b} protein in both the normal (Figure 5B, lanes 5 and 6) or reversed (Figure 5B, lanes 7 and 8) orientations. Similarly, insertion of the same fragment upstream of the V gene segment (on the 5′ side of the transcriptional promoter) in either the normal (Figure 5B, lanes 9 and 10) or the reversed (Figure 5B, lanes 11 and 12) orientation also restored the expression of γ_{2b} protein to normal levels.

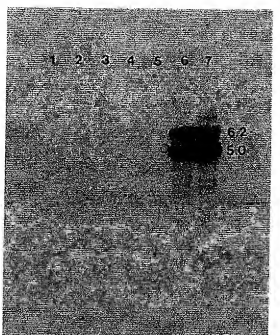

Figure 4. Southern Gel Blotting Analysis of Plasmid DNA Sequences in Transfected Cell Lines

High molecular weight DNA (10 μg per lane) from J558L cells (lane 1), or cell lines transfected with plasmid pSV-γ_{2b}VC (lanes 2 and 3), plasmid pSV-γ_{2b}3′RΔ1 (lanes 4 and 5), or plasmid pSV-γ_{2b}3′RΔ2 (lanes 6 and 7) was digested with Hind III, electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized to nick-translated pSV2*gpt* DNA. The positions of the two Hind III fragments (6.2 kb and 5.0 kb), common to all the transfected plasmid DNAs (both of which hybridize to the pSV2*gpt* probe), are indicated.

These results clearly demonstrate that the intron sequences deleted in the 3′RΔ2 mutant plasmid have a direct effect on transcription in a manner that is analogous to the viral enhancers. They also show that the enhancer function does not require the expression of these sequences in the γ_{2b} gene primary transcript, because movement of the $X_{2/3}$ fragment outside of the transcription unit (i.e., the Xba I$_1$ site) had no effect on its ability to function.

Tissue Specificity of the Immunoglobulin Enhancer Element

The rearranged γ_{2b} gene used in these studies is also accurately transcribed in mouse fibroblasts (Ltk$^-$ cells)

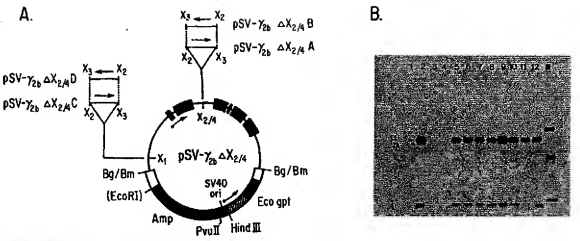

Figure 5. Enhancement of γ_{2b} Gene Expression by a 1 kb Intron Fragment

(A) Restriction map of plasmid pSV-$\gamma_{2b}\Delta X_{2/4}$. This plasmid was constructed from plasmid pSV-γ_{2b}VC (shown in Figure 1) by removing two Xba I fragments from the γ_{2b} gene intron (from the X_2 to the X_4 sites in Figure 1). Derivatives of plasmid pSV-$\gamma_{2b}\Delta X_{2/4}$, labeled A through D, contain inserts of the 1 kb $X_{2/4}$ fragment in the sites indicated and the orientation (relative to transcription) is shown with an arrow. (B) Expression of γ_{2b} heavy chain in cells transfected with the plasmids shown in (A). Analysis was carried out as described in Figure 2. Cell lines tested were J558L (lane 1), and those transfected with plasmid pSV-γ_{2b}VC (lane 2), plasmid pSV2-$\gamma_{2b}\Delta X_{2/4}$ (lanes 3 and 4), plasmid pSV-$\gamma_{2b}\Delta X_{2/4}$A (lanes 5 and 6), plasmid pSV-$\gamma_{2b}\Delta X_{2/4}$B (lanes 7 and 8), plasmid pSV-$\gamma_{2b}\Delta$-$X_{2/4}$C (lanes 9 and 10), and plasmid pSV-$\gamma_{2b}\Delta X_{2/4}$D (lanes 11 and 12).

cotransfected with the herpes virus tk gene and plasmid ppL-γ_{2b}VC (Gillies and Tonegawa, 1983). The level of γ_{2b} gene expression in these cells was found to be proportional to the number of transfected genes, but is at least two orders of magnitude less per gene copy than in myeloma cells. Thus it is likely that the enhancer element, described above, does not function in nonlymphoid cells.

In order to compare the levels of γ_{2b} gene expression in fibroblasts, with and without the immunoglobulin enhancer, we first made a deletion mutant, ppL-$\gamma_{2b}\Delta X_{2/3}$, lacking these sequences (Figure 6A). We then modified the plasmids so that a high copy number of γ_{2b} genes would be integrated into the transfected L cells and increase the expression of the γ_{2b} gene to an easily detectable level. This was done by inserting a truncated *tk* gene (a 2.3 kb Eco RI fragment containing only limited 5′ upstream sequences) into both the wild type and mutant plasmids. Transformation to the *tk*$^+$ phenotype with this fragment requires the transfer of multiple plasmid copies into cells, thus another gene on the same plasmid would also be present at a high copy number in *tk*$^+$ transformants (our unpublished results).

Plasmids ppL-γ_{2b}-TK and ppL-$\gamma_{2b}\Delta X_{2/3}$-TK were introduced into mouse Ltk$^-$ cells and the *tk*$^+$ transformants (approximately 50 individual clones) were pooled, grown in mass culture, and tested for the presence of γ_{2b} DNA sequences. As seen in Figure 6B, each transfected cell line contained comparable numbers of tandem, head-to-tail oligomers of either plasmid. Control experiments (not shown) indicate that individually cloned cell lines also contain the same number (about 15 copies per cell) of transfected plasmid DNA. Apparently the copy number is determined by the level of expression of the *tk* gene which, in this case, has been reduced considerably by the deletion of the upstream sequences. To compensate for the low level of expression, multiple copies of the *tk* gene are required for *tk* transformation. This, then, is analogous to the results with pSV2*gpt* vectors described above.

We compared the expression of the normal and mutant γ_{2b} heavy chain genes in these cell lines by Northern gel blotting analysis of total cell RNA. As seen in Figure 6C, the steady-state level of γ_{2b} mRNA is not affected by the deletion of the immunoglobulin enhancer. We concluded that the low level expression of the heavy chain gene in L cells is a result of the fact that this enhancer element is functional only in lymphoid cells.

Additional experiments have been carried out to test the tissue specificity of the immunoglobulin enhancer. We constructed a derivative (pSER) of plasmid pSV2*gpt* lacking most of the SV40 72 bp repeat sequence (see Experimental Procedures). When this plasmid is used to transfect either mouse L cells or J558L myeloma cells, the transformation frequency (relative to that of plasmid pSV2*gpt*) is lowered by more than a factor of 20 (from 2×10^{-3} to 10^{-4} in L cells and from 3×10^{-4} to 10^{-5} in J558L myeloma cells—Table 1). When the 1 kb $X_{2/3}$ fragment containing the immunoglobulin enhancer is inserted into the Eco RI site of plasmid pSER, the transformation frequency is restored to the level of plasmid pSV2*gpt*, but only in myeloma cells. There is no effect on the transformation frequency of plasmid pSER in L cells (Table 1). Thus the enhancing effect on the heterologous SV40 promoter (which controls the Eco*gpt* gene) is also tissue-specific.

Using this same transformation assay we tested smaller restriction fragments for enhancer activity. A 140 bp Pvu II–Dde I fragment (see Figure 1), containing some of the

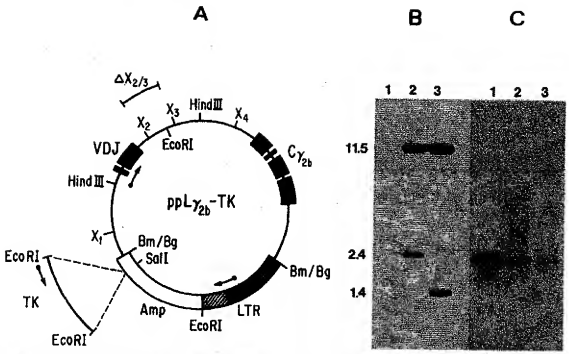

Figure 6. Expression of γ_{2b} mRNA in Mouse L Cells Transfected with Plasmids pp$L\gamma_{2b}$-*tk* and pp$L\gamma_{2b}\Delta X_{2/3}$-*tk*

(A) Restriction map of the plasmids used for transfection. Plasmid pp$L\gamma_{2b}$-*tk* was constructed by inserting a 2.3 kb fragment of the herpes virus *tk* gene into the unique Sal I site of plasmid pp$L\gamma_{2b}$VC (Gillies and Tonegawa, 1983). The direction of transcription of the *tk* gene (arrow) is opposite that of the γ_{2b} gene. The sequences deleted in plasmid pp$L\gamma_{2b}\Delta X_{2/3}$-*tk* are indicated. (B) Southern gel blotting analysis of DNA from L *tk*$^-$ cells (lane 1), and cells transfected with plasmid pp$L\gamma_{2b}$-*tk* (lane 2), or plasmid pp$L\gamma_{2b}\Delta X_{2/3}$-*tk* (lane 3). DNA was digested with Hind III and hybridized to nick-translated γ_{2b} DNA (the 9 kb Bgl II fragment used for plasmid construction). (C) Northern gel blotting analysis of total cell RNA from MOPC 141 myeloma cells (lane 1), and cells transfected with plasmid pp$L\gamma_{2b}$-*tk* (lane 2), or plasmid pp$L\gamma_{2b}\Delta X_{2/3}$-*tk* (lane 3). Nick-translated $C\gamma_{2b}$ probe (Gillies and Tonegawa, 1983) was used for hybridization.

sequences deleted in plasmid pSV-γ_{2b}3′RΔ2, was found to increase the transformation frequency of plasmid pSER by 20-fold in J558L cells but not in mouse L cells (Table 1). Thus we have localized the immunoglobulin enhancer sequence to this portion of the $X_{2/3}$ fragment.

DNA Sequences in the Heavy Chain Gene Intron Resemble Viral Enhancers

Weiher et al. (1983) have suggested that the sequence 5′ GTGG$\overset{AAA}{TTT}$G3′ (where $\overset{A}{T}$ means either A or T appears at that position) represents a crucial core element common to all of the known viral enhancers. Sequence analysis of the $X_{2/3}$ fragment (the 1 kb fragment shown to have enhancer activity—Figure 5) shows that such a sequence is located in the region that is deleted in plasmid pSV-γ_{2b}3′RΔ2 (but not in the 3′RΔ1 mutant) and is present in the 140 bp Pvu II–Dde I fragment. In fact, the sequence 5′GTGGTTT(T)GAA-3′ is present as a closely spaced repeat (Figure 7), oriented in the direction of transcription. The first eight nucleotides of this sequence are also found upstream of the tandem repeat, but oriented in the opposite direction.

Figure 8 shows a comparison of several viral sequences shown to have enhancer activity and the repeat sequences

Table 1. Transformation Frequency of pSV2*gpt* and Derivative Plasmids in J558L Myeloma Cells and L Cells

	Cell Type	
Plasmid	J558L	L Cell
pSV2*gpt*	3×10^{-4}	2×10^{-5}
pSER	8×10^{-4}	1×10^{-4}
pSER-$X_{2/3}$	4×10^{-4}	1×10^{-4}
pSER-$X_{2/3}$(140)	2×10^{-4}	9×10^{-4}

Cells were transfected by protoplast fusion and plated at 10^4 cells per well and 2×10^5 cells per well (J558L) or at 10^4 and 10^5 cells per 100 mm dish (L cells). Selective medium containing mycophenolic acid (8 μg/ml for J558L or 25 μg/ml for L cells) was added at 48 hr and colonies were counted at 10 days (J558L) or at 14 days (L cells). Derivatives of plasmid pSER were constructed by inserting (blunt-end ligating) either the 1 kb $X_{2/3}$ fragment or a 140 bp Pvu II–Dde I [$X_{2/3}$(140)] fragment (see Figure 1) into the Eco RI site.

in the γ_{2b} gene intron. Sequences contained in the Moloney sarcoma virus (MSV) 73 bp repeat sequence appear to be most similar to the immunoglobulin sequence, especially on the 5′ side of the first "core" repeat. The "core" sequence of polyoma virus was most similar to the second "core" repeat, as both contain an additional T residue.

Also shown are two sequences, present in the immu-

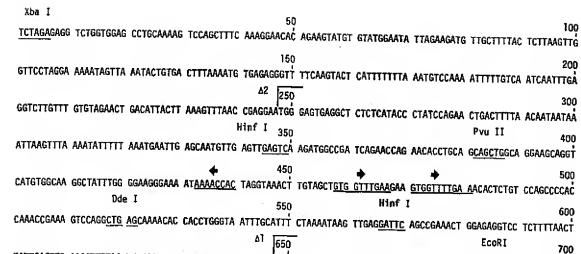

Figure 7. Nucleotide Sequence of the 1 kb X_{ba} Fragment

DNA sequencing was carried out according to standard procedures (Maxam and Gilbert, 1980). The sequences deleted in plasmids pSV-γ_{2a}3′RΔ1 and pSV-γ_{2a}3′RΔ2 are indicated. The underlined sequences are those similar to the "core" elements common to most viral enhancers (Weiher et al., 1983). Arrows indicate the orientation of the immunoglobulin "core" elements relative to the direction of γ_{2b} mRNA transcription.

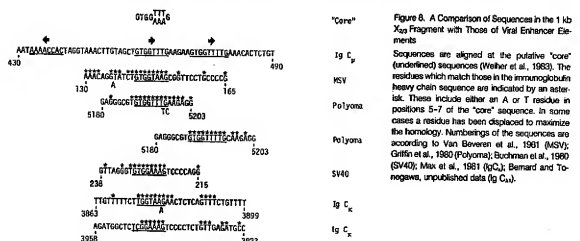

Figure 8. A Comparison of Sequences in the 1 kb X_{ba} Fragment with Those of Viral Enhancer Elements

Sequences are aligned at the putative "core" (underlined) sequences (Weiher et al., 1983). The residues which match those in the immunoglobulin heavy chain sequence are indicated by an asterisk. These include either an A or T residue in positions 5–7 of the "core" sequence. In some cases a residue has been displaced to maximize the homology. Numberings of the sequences are according to Van Beveren et al., 1981 (MSV); Griffin et al., 1980 (Polyoma); Buchman et al., 1980 (SV40); Max et al., 1981 (γC_μ); Bernard and Tonegawa, unpublished data ($\gamma_2 C_\kappa$).

noglobulin light chain gene intron, that resemble an enhancer element. The existence of such a sequence near the C_κ gene segment is rendered plausible by its proximity to a DNAase I hypersensitive site (Parslow and Granner, 1982). In addition, studies using transfected genes suggest that deletions in this region reduce transcription from the V promotor (V. O. and S. M., unpublished data). A similar core-like sequence is also present near the constant portion of the λ_1 light chain gene, but there is yet no evidence showing that this is part of an enhancer element.

Discussion

Evidence for an Enhancer Element in the Intron of a Heavy Chain Immunoglobulin Gene

Sequences contained in the major intron between the functionally rearranged VDJ and C exons of a heavy chain immunoglobulin gene were shown to be essential for its high level expression in transfected myeloma cells. Although the deletion of these sequences decreases the level of expression in transfected cells 20 times (Figure 2), the actual reduction is probably about 400 times per gene copy. We have tested whether these sequences are analogous to viral enhancer elements. In addition to increasing the level of transcription from homologous promoters, viral enhancers also increase transcription from many heterologous, viral or nonviral promoters. This enhancing activity is independent of the orientation of the enhancer element, relative to the direction of transcription, and is independent of its position as long as the distance between the enhancer and promoter is within several kilobases (Banerji et al., 1981; Moreau et al., 1981; Wasylyk et al., 1983).

These properties also apply to the sequences contained in the heavy chain immunoglobulin gene. Enhancement of the heterologous SV40 promoter occurs when the intact γ_{2b} heavy chain gene is present in plasmid pSV2*gpt* and a low copy number of the recombinant plasmid is sufficient for *gpt* transformation. When the γ_{2b} gene intron sequences are removed, the plasmid copy number increases dramatically to compensate for the decreased expression of the *gpt* gene from the SV40 promoter (Figure 4).

We also found that DNA fragments from the γ_{2b} gene intron can substitute for the SV40 enhancer in plasmid pSV2*gpt* (Table 1). This transformation assay is based on the ability of the DNA fragments, located more than 2 kb away from the SV40 promoter, to enhance the transcription of the *gpt* gene and thereby increase the transformation frequency. Using this method we have shown that most of the enhancing activity can be localized to a 140 bp fragment. The nucleotide sequence in this region contains a repeat sequence which closely resembles the "core" nucleotides found in most viral enhancers (Figure 8).

Finally, we demonstrated that this intron sequence maintains its ability to stimulate transcription of the heavy chain gene when it is moved outside of the γ_{2b} transcription unit (5′ of the mRNA start site) and when its orientation is reversed (Figure 5). These results show that the immunoglobulin intron sequence is an enhancer element and has properties in common with those of viral origin, even though the latter are generally located on the 5′ side of their cognate transcriptional promoters.

The Role of Transcriptional Enhancement in the Regulation of Immunoglobulin Gene Expression

The creation of active immunoglobulin genes through somatic recombination has been studied in detail (reviewed by Tonegawa, 1983), but the mechanism by which this activation is brought about has been a major problem of molecular immunology. The observation that the C_κ and C_γ gene segments are transcriptionally active in lymphoid cells, prior to V–J or V–D–J joining (Kemp et al., 1980; Van Ness et al., 1982), provided the first evidence that sequences downstream of the V gene segment promoter might affect the transcription of the functionally rearranged gene.

The mechanism of this activation can now be explained, at least for the heavy chain gene, by our identification of an enhancer element between the J_H and $C_{\gamma 2b}$ gene segments of a functionally rearranged gene. This site corresponds to the J_H–C_μ region of germ-line DNA (see Figure 9). Following VDJ-joining, which occurs before B cells encounter antigens, this enhancer (which would now be part of the major intron of the functionally rearranged μ

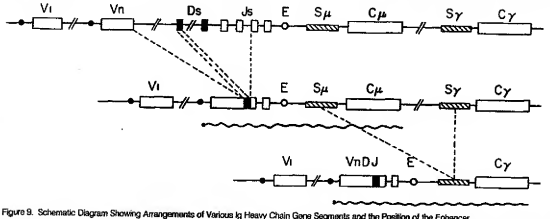

Figure 9. Schematic Diagram Showing Arrangements of Various Ig Heavy Chain Gene Segments and the Position of the Enhancer

Top, middle, and bottom arrangements correspond to germ-line DNA; μ chain-positive, preswitch B cell DNA; and γ chain-positive, postswitch plasma cell DNA, respectively. The enhancer element (O) is located between the J_H segments and C_μ segments in the germ-line DNA and becomes part of the major intron in the active μ chain gene upon V–D–J joining. The same enhancer element is retained in the major intron of the active γ chain gene, which is created by a switch recombination from the μ gene. S_μ and S_γ refer to the regions in which switch recombinations occur. The wavy lines and the large filled circles (●) represent the primary transcripts and the promoters, respectively. The small filled circles (•) represent the 5′ caps of the RNA molecules.

chain gene) activates the promoter of the rearranged V gene segment. In this way only a single V gene segment (out of several hundred) would be transcriptionally active, and only after functional rearrangement had occurred.

Subsequent to the encounter with antigens and stimulation by T cells, a second type of rearrangement (switch recombination) occurs in heavy chain genes and results in the replacement of the C_μ coding sequence (Figure 9) with those of the other heavy chain classes and subclasses (Maki et al., 1980; Kataoka et al., 1980; Sakano et al., 1980; Davis et al., 1980). In order to function after class switching, the immunoglobulin enhancer would have to be located upstream of the switch region, otherwise it would be deleted along with the C_μ coding sequence. This is in fact the case since its location is more than 1 kb upstream of any known switch sites within the S_μ region. Thus as we have shown for the MOPC 141 γ_{2b} gene, it is likely that the same enhancer is used for the expression of all heavy chain classes following switch recombination, although it is possible that additional regulatory elements may be associated with the individual C region gene segments.

Evidence for Other Cellular Enhancer Elements

The data presented in this report represents the first clear demonstration of an enhancer element being associated with a defined cellular gene. The possibility that enhancers are present in cellular DNA has already been suggested by others. For example, Conrad and Botchan (1982) isolated human DNA sequences which hybridized to the region of SV40 DNA spanning the origin of replication. One of these sequences was found to enhance the efficiency of *tk* transformation in an orientation-independent manner and thus resembles the viral enhancer elements. Furthermore, this DNA sequence cross-hybridized with many sequences in human DNA, suggesting that a family of such elements exists.

Rosenthal and Khoury (personal communication) have likewise isolated a human DNA sequence by virtue of its cross-hybridization with a portion of BK virus DNA. In this case, however, the sequence appears to be unique in the human genome even though it contains repeating 21 bp elements. These repeat elements show some homology to the BK virus enhancer region but appear to be about 8 times less active when tested for enhancing activity in the CAT assay of Gorman et al. (1982).

Thus it is likely that enhancer elements might serve as a general mechanism for gene regulation in eucaryotes. The association of such elements with specific genes is currently being studied in several systems. In addition to our demonstration of an enhancer element in the heavy chain immunoglobulin gene, two of us (V. O. and S. M.) have found that sequences near the C_κ gene segment are essential for the high level expression of κ chains in transfected myeloma cells. In this case, however, the functional similarity of this sequence element to the viral enhancers is less clear.

The use of enhancer elements as regulators of gene expression may not be confined to higher eucaryotic systems. There is evidence (L. Guarente, personal communication) that the yeast iso-1-cytochrome c gene is activated by heme and that sequences upstream of the gene are essential for this effect. Furthermore, the inversion of this activator sequence did not affect the inducibility of expression. This strongly suggests that this region is not simply a component of the transcriptional promoter.

Tissue-specific Enhancer Elements and Their Possible Role in Cell Differentiation

The most interesting property of the immunoglobulin enhancer is its tissue specificity. The MSV and SV40 viral enhancers have been shown to have a certain degree of host cell specificity (Lalmins et al, 1982). This effect may also be explained in terms of tissue specificity because the two cell types used for the comparison were derived from different tissues. The immunoglobulin enhancer, on the other hand, functions at a high level in a lymphoid (myeloma) cell type but not at all in another cell type (fibroblast) of the same species. It is likely that this specificity is the result of a factor (or factors), present only in lymphoid cells, which regulates immunoglobulin expression during B cell ontogeny.

At early stages of B cell development, μ heavy chains are expressed at a low level (Levitt and Cooper, 1980). After the B cell encounters antigen and interacts with regulatory T cells, terminally differentiated plasma cells appear and produce very high levels of immunoglobulin (Schibler et al, 1978). The quantitative differences in the level of immunoglobulin gene expression at different stages of B cell development suggest that the enhancer function may be stage-specific. It is also possible that multiple regulatory elements are contained within this enhancer region and that increased levels of expression result from the combined effect of individual enhancers.

Another possible example of a tissue-specific enhancer element has been described in the polyoma virus system (Katinka et al., 1980; Fujimura et al., 1981; Fujimura and Linney, 1982). It was shown that polyoma mutants that acquire the ability to replicate in the otherwise refractory F9 embryonal carcinoma cells contain point mutations and, in some cases, tandem duplications near the region of polyoma DNA which was shown (de Villiers et al., 1981) to have enhancer activity. This result suggests that certain DNA sequences are recognized as an enhancer in cells permissive for polyoma virus but that sequences located at an adjacent site are recognized (after being mutated) in embryonal cells. Thus it appears that polyoma, like immunoglobulin genes, may contain multiple regulatory elements within their enhancer regions.

The mechanism of tissue-specific enhancer is not known and this simply reflects our present ignorance of the mechanism of enhancers in general. Clearly though, the sequence differences and similarities between the various enhancers strongly suggest that specific regulatory proteins recognize these sites. In fact, the glucocorticoid

receptor protein, which binds to the promoter region of mouse mammary tumor virus (MMTV), may be an example of an enhancer binding protein (K. Yamamoto, personal communication). A sequence upstream of the MMTV promoter, essential for hormone responsiveness, was shown to enhance the herpes *tk* gene in an orientation-independent manner and to bind the hormone receptor.

It is tempting to speculate that the presence or absence of such enhancer binding proteins determines whether or not an enhancer functions in a given cell type. Furthermore, a particular enhancer binding protein might recognize many different but related sequences to a greater or lesser extent (or bind with different affinities). In this way it would be possible to activate many individual genes and to express them at different levels. Alternatively, the level of expression of a gene that is controlled by an enhancer may be determined by the distance between this element and the promoter site. It seems likely that some or all of these mechanisms of gene regulation function during the process of cellular differentiation. In this way the expression of multiple genes could be controlled (coordinately expressed) by a relatively small number of regulatory proteins.

Enhancers as Activators of Cellular Oncogenes

One striking case for the role of enhancers in tumorigenesis was provided by studies of avian leukosis virus (ALV) induced chicken B cell lymphomas. In such lymphomas, ALV DNA was found to be integrated adjacent to the *c-myc* gene (Payne et al., 1981), the cellular counterpart of the transforming sequences from the MC29 group of defective retroviruses (Sheiness and Bishop, 1979). Although it was first thought that *c-myc* was activated by a promoter-insertion mechanism (Hayward et al., 1981), it was later shown that ALV insertions could occur in the opposite orientation or downstream of the *c-myc* gene (Payne et al., 1982). Thus the ALV enhancer element was responsible for the increased level of *c-myc* expression and, presumably, for oncogenic transformation.

The role of cellular enhancer elements in the activation of oncogenes has also been suggested by recent findings (reviewed by Klein, 1983). Many murine and human tumors of lymphoid origin have been shown to contain chromosomal translocations in which an oncogene (*c-myc*) has been rearranged to an immunoglobulin C region gene segment. The majority of rearrangements in human Burkitt lymphomas were found to occur at the C_κ region while those in mouse plasmacytomas occur at the C_α region. While the results we have described could account for the activation of *c-myc* in some human C_α rearrangements (by analogy to the murine C_α enhancer), the results in the murine system are somewhat unclear. It has not been ruled out, however, that the murine C_α gene segment contains an additional enhancer element.

We are currently investigating the activation of *c-myc* by sequences contained near the *c-myc*-C_γ junction in human DNA. It will be interesting to compare the sequences in this region with those that we showed to contain enhancer activity. Sequences that have been conserved through evolution may also help to identify the critical components of this regulatory element.

Experimental Procedures

Cell Culture and Transfection

The myeloma cell line, J558L, is a heavy chain loss variant of J558 and synthesizes λ light chains (Oi et al., 1983). Cells were grown in Dulbecco's modified Eagle's medium (MEM) containing 10% fetal calf serum. J558L cells were transfected by a modification of the protoplast fusion technique (Sandri-Goldin et al., 1981). Approximately 2×10^8 cells (grown to a density of 4 to 6×10^5 cells/ml) were washed once with serum-free MEM, collected by centrifugation (5 min. at 500 g), and suspended by gentle pipetting in the protoplast suspension (approximately 2×10^8 protoplasts in 4 ml). The cell-protoplast suspension was transferred to a 60 mm dish and centrifuged at 1500 g for 7 min. After gentle aspiration of the supernatant, 1.5 ml of 50% PEG-1500 (in serum-free MEM and prewarmed to 37°C) was added and the dish was spun at 500 g until 90 sec had elapsed from the time of PEG addition. Cells were resuspended by gently pipetting in two 5 ml washes of prewarmed, serum-free MEM which were added to 15 ml of MEM in a 50 ml centrifuge tube. Following centrifugation at 500 g for 5 min, cells were resuspended in growth medium containing kanamycin (100 μg/ml) and plated in 96-well dishes at two densities: 1×10^4 cells per well and 2×10^5 cells per well. After 48 hr selective medium (Oi et al., 1983) was added.

Plasmid Constructions

Plasmid pSV-γ_{2b}VC, containing the γ_{2b} gene from myeloma MOPC 141, was constructed by inserting a 9 kb Bgl II fragment (Gillies and Tonegawa, 1983) from phage clone M141-pC1 (Sakano et al., 1980) into the unique Bam HI site of plasmid pSV2gpt(Pl). This latter plasmid was constructed by mutating the Eco RI site of plasmid pSV2gpt (Mulligan and Berg, 1980). The transcription orientation of the γ_{2b} gene is opposite that of the *gpt* gene (Figure 1).

Plasmids pSV-γ_{2b}3'RA1 and pSV-γ_{2b}3'RA2 were constructed by digesting Eco RI-cut pSV-γ_{2b}VC DNA with exonuclease *Bal* 31 (1 U/μg of DNA) at 23°C for 2 or 4 min and recircularizing the products with T4 DNA ligase. The extent of the deletions were determined by restriction analysis and DNA sequencing.

Plasmid pSV-$\gamma_{2b}\Delta X_{bh}$ was constructed by first digesting plasmid pSV-γ_{2b}VC DNA with Bgl II and then partially digesting with Xba I. The 6.5 kb partial digestion product extending from the unique Bgl II site to the Xba I site (X_d) on the 3' side of the VDJ exon (clockwise on the map in Figure 1) and the 5.1 kb complete digestion product extending from the Bgl II site, counterclockwise to the Xba I site (X_a) on the 5' side of the $C\gamma_{2b}$ coding region, were gel purified and ligated. The resulting plasmid, pSV-$\gamma_{2b}\Delta X_{bh}$, was used for the experiment shown in Figure 5. Derivatives of this plasmid (A–D) were constructed by partially digesting with Xba I, treating the DNA with calf intestine alkaline phosphatase, purifying linear full-length DNA, and ligating the products with the 1 kb Xba I fragment extending from the X_d to X_b sites (X_{bh} fragment in Figure 1). The site of insertion and the orientation of the X_{bh} fragment were determined by restriction analysis.

Plasmid pSER was constructed by digesting plasmid pSV2gpt DNA with Sph I and Pvu II and removing the 3' protruding bases with T4 DNA polymerase (O'Farrell, 1981). The two blunt ends were then ligated to produce a selectable plasmid vector which no longer contains the SV40 enhancer sequence.

Analysis of Transfected Cells

Approximately 10 days after transfection, the cells contained in a single well (from 5 to 10 independent clones) were harvested and grown in mass culture for analysis of protein synthesis and the steady-state level of γ_{2b} mRNA (Gillies and Tonegawa, 1983). Four such pools were analyzed for each plasmid tested as well as subclones obtained by limiting dilution.

Protein synthesis was measured by labeling 5×10^6 cells for 1 hr with ^{35}S-methionine (50 μCi/ml) and analyzing immunoprecipitated cell extracts as described (Gillies and Tonegawa, 1983). Secretion of immunoglobulin

was measured by labeling approximately 2×10^6 cells for 16 hr in 50 μl of normal growth medium containing ^{35}S-methionine (25 μCi/ml). Immunoglobulin was then immunoprecipitated from culture supernatants.

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Cell-Type Specificity of Immunoglobulin Gene Expression is Regulated by at Least Three DNA Sequence Elements

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Summary

The regulation of cell-type specificity of immunoglobulin (Ig) μ heavy chain (H) gene expression was examined by introducing various hybrid genes containing Ig gene sequences combined with portions of a tissue-nonspecific transcription unit into lymphoid and nonlymphoid cells. Replacing the lymphocyte-specific IgH enhancer with a viral enhancer did not affect tissue specificity of μ Ig gene expression. We identified two new regulatory regions that provide transcriptional tissue specificity. First, the V_H promoter region between position −154 and +57 was shown to direct lymphocyte-specific transcription of a bacterial *gpt* gene, even in the presence of a viral enhancer. Second, μ intragenic sequences, lacking the IgH enhancer, were found to regulate the level of accumulated Ig transcripts in a tissue-specific fashion. These results demonstrate that tissue specificity of Ig gene expression is not solely regulated by the enhancer but that the promoter, and as yet undefined intragenic sequences, contain lymphoid-specific regulatory information.

Introduction

Immunoglobulin (Ig) genes are expressed specifically in cells of the B lymphocyte lineage. Very early in B cell differentiation the heavy chain (H) locus becomes transcriptionally active, and the variable (V) gene segment is assembled from its various components by DNA rearrangement (Tonegawa, 1983). DNA rearrangement is accomplished in two steps, D-to-J_H and V_H-to-DJ_H joining, with the first rearrangement sometimes occurring in T cells (Kurosawa, 1981; Alt et al., 1984). Somatic recombination is not required for the transcriptional activation of the heavy chain gene because even incompletely rearranged or unrearranged genes are transcribed from pseudopromoters 5′ to the constant (C) region (Kemp et al., 1980; Alt et al., 1982; Nelson et al., 1983). In completely rearranged genes the promoter 5′ to the V gene segment is used (Clarke et al., 1982). Transcription of the heavy chain gene is tissue-specific and restricted to B and T lymphocytes, implying that lymphoid cells contain specific *trans*-acting factors required for heavy chain gene activation. Because complete V_HDJ_H joining is restricted to B cells, only these cells can synthesize functional heavy chains.

Within the B lymphoid cell lineage, expression of the heavy chain gene is cell-type-specific. In early stage cells, termed pre-B cells, transcriptional activation and rearrangement of the heavy chain gene occurs (Maki et al., 1980; Alt et al., 1981). In mature B cells, light chain genes are expressed, and surface immunoglobulins are synthesized (Alt et al., 1980; Rogers et al., 1980). In both cell types, the level of accumulated Ig mRNA is low (Perry and Kelley, 1979). When B cells encounter antigen, the cells mature into terminally differentiated plasma cells, and the amount of accumulated Ig mRNA is increased 30- to 100-fold (Perry and Kelley, 1979). Most of this dramatic increase in Ig gene transcript accumulation is due to post-transcriptional regulation because the rate of transcription in plasma cells is accelerated only 2- to 5-fold over the rate in B cells (Mather et al., 1984).

Gene transfer experiments, in which cloned rearranged wild-type or in vitro mutated Ig genes have been introduced into terminally differentiated lymphoid and nonlymphoid cells, have pointed to certain mechanisms of Ig gene regulation. Two regulatory elements have been described that are crucial for the expression of Ig genes in myeloma cells, the tumor cell counterpart of plasma cells. One element, residing in the intron between the variable and constant regions of both heavy and κ light chain genes, acts as a transcriptional enhancer (Banerji et al., 1983; Gillies et al., 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984; Bergman et al., 1984; Potter et al., 1984). Because the heavy chain enhancer is inoperative in nonlymphoid cells, it has been suggested that this regulatory element controls the tissue-specific expression of Ig genes (Banerji et al., 1983; Gillies et al., 1983). Another sequence element located about 30 bp upstream of the TATA box was found to be required for κ gene transcription in myeloma cells (Falkner and Zachau, 1984; Bergman et al., 1984).

To investigate which sequence elements are regulating tissue specificity and modulation of Ig gene transcription during B cell differentiation, we have dissected the heavy chain transcription unit into enhancer, promoter, and intragenic sequences and tested these elements individually for their contribution to regulation of Ig gene expression. We show here that each of the three regulatory elements can confer tissue specificity to heterologous transcription units. The heavy chain enhancer is required for Ig gene transcription, but it can be replaced with a viral enhancer without affecting the tissue specificity of gene expression. The V_H promoter region and the μ intragenic sequences also control the differential Ig gene expression in lymphoid versus nonlymphoid cells.

Results

To study the expression of Ig heavy chain genes in cultured mouse cells, a functionally rearranged μ gene containing a V_H gene segment isolated from the hybridoma 17.2.25 (Loh et al., 1983; Grosschedl et al., 1984) was inserted into a short-term expression vector (Figure 1A). The vector contained the early region of polyoma virus to in-

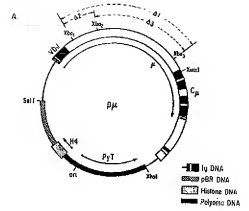
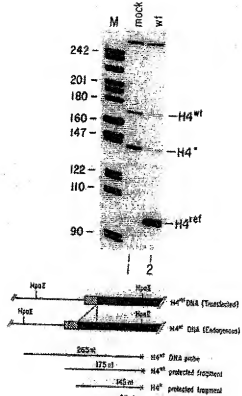


Figure 1. Structure and Expression of the Plasmid pμ in Myeloma MPC11 Cells

(A) Map of the plasmid DNA. The μ gene with its rearranged V_H gene segment isolated from the hybridoma 17.2.25 is represented by filled bars (exons), open bars (introns), and hatched bars (noncoding sequences); the 5'- and 3'-flanking regions of the μ gene are shown as lines. The polyoma virus (Py) early region is depicted as a narrowed filled bar. The Py early region includes the origin of replication (ori) and DNA sequences encoding T antigens. Vector pBR322 sequences and H4 gene sequences are drawn hatched and dotted, respectively. The transcriptional polarities of the genes are indicated by arrows. The structure of deletions in the $V_HDJ_HJC_H$ introns are shown. The deleted DNA sequences are indicated by dashed lines extending between Xba I cleavage sites.



(B) S1 nuclease mapping of specific V_H17.2.25 RNA 5' ends. Total RNA (20 μg) from myeloma MPC11 cells transfected with $p\mu$ wild-type DNA (lane 1), mock-transfected cells (lane 3), or total 17.2.25 hybridoma RNA (0.2 μg) (lane 2) were hybridized to the 5' end-labeled DNA probe shown (bottom) and digested with S1 nuclease as described in Experimental Procedures. The protected fragments were separated by electrophoresis and revealed by autoradiography. Lane M, ^{32}P labeled Hpa II-digested pBR322 DNA as size marker. The sizes are indicated in base pairs. The arrow indicates the 57 nucleotide major protected fragment. (Bottom) the structure of the 5' end of the μ gene is shown. The leader coding sequences are drawn as a filled bar; intron sequences are shown as an open bar and 5' noncoding sequences as a hatched bar. The structures and sizes of the single-stranded DNA probe and the protected DNA fragment are shown.

(C) Analysis of H4 transcripts. Total RNA (5 μg) from MPC11 cells transfected with $p\mu$ DNA (lane 2) or mock-transfected cells (lane 1) was analyzed for the presence of H4 histone transcripts by S1 nuclease mapping. The position of the 99 nucleotide fragment protected by transcripts of the H4 reference gene is indicated as $H4^{ref}$. Two other fragments of 175 and 145 nucleotides mapped to the initiation site of transcription and to the ATG initiation codon of the wild-type H4 gene. The 175 nucleotide protected fragment corresponded to transcripts from the endogenous H4 gene ($H4^{wt}$) and the 145 nucleotide fragment corresponded to RNA from endogenous H4 variant genes ($H4^{*}$) that contain sequence homology to the coding region of the DNA probe. The structure of the $H4^{wt}$ and $H4^{ref}$ genes is shown at the bottom. The coding regions of the wild-type and the mutant H4 genes are depicted as filled bars. The deletion in the mutated reference gene is indicated by dashed lines. The DNA probe used for S1 nuclease mapping consisted of a 265 nucleotide Hpa II fragment that had been isolated from the wild-type H4 gene. The structure and sizes of the protected DNA fragments are shown.

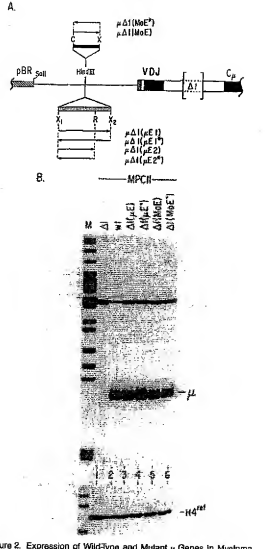


Figure 2. Expression of Wild-Type and Mutant μ Genes in Myeloma MPC11 Cells and NIH/3T3 Fibroblasts

(A) Structure of enhancer insertion mutations. The μΔ1 gene, which is lacking all DNA sequences between the Xba I_1 and Xba I_2 sites in the J_H/C_μ intron including the IgH enhancer (Figure 1A), is shown. Various DNA fragments containing either the Ig enhancer or the M-MuLV (Mo) enhancer were inserted into the Hind III site 5′ of the promoter. The enhancer fragments are drawn as arrows indicating their relative orientation. The restriction sites flanking the DNA fragments are abbreviated as X, for Xba I; C, for Cla I; and R, for Eco RI.

(B) Expression of the wild-type and mutant μ genes in MPC11 myeloma cells. Total RNA (20 μg) from transfected MPC11 cells was hybridized to the 5′ end-labeled μ DNA probe, shown in Figure 1C, and was digested with S1 nuclease prior to electrophoretic separation. Lane M, Hpa II-cleaved pBR322 DNA as size marker. The position of the 57 nucleotide fragment protected by 5′ terminal μRNA sequences is indicated as μ. The 155 nucleotide upper band corresponds to the full-length DNA probe.

(C) Transcriptional activity of the wild-type and mutant μ genes in NIH/3T3 fibroblasts. Total RNA from NIH/3T3 cells transfected with various μ gene constructs (see Figures 1A and 2A) was assayed for the presence of specific μ and $H4^{rat}$ transcripts by S1 nuclease mapping as described in Figure 1B and 1C. Lane M, Hpa II-cleaved pBR322 DNA. The position where a DNA fragment protected by μmRNA would migrate is indicated as μ. The 155 nucleotide fragment visible in all lanes corresponds to the full-length DNA probe.

crease the copy number of the linked genes and thus the amount of accumulated RNA in the transfected cells (Queen and Baltimore, 1983). Control experiments with plasmids lacking the polyoma virus DNA segment, as well as the total absence of transcription when Ig regulatory sequences were deleted, suggested that the polyoma DNA segment did not perturb the regulation and expression of the μ gene in our construct. To control for the effects of sequence alterations in the μ gene, we included in the construct a mouse H4 histone gene, which has a small transcription unit and no obvious tissue- or cell-type specificity (Seifer-Tynns and Birnstiel, 1981).

The complete DNA construct, termed $p\mu$ (Figure 1A), was transfected into MPC11 myeloma cells. Total RNA was isolated after 48 hr and analyzed for the presence of specific μ transcripts by S1 nuclease mapping of RNA 5′ ends (Figure 1B). Protected fragments, about 57 nucleotides in length, were generated (Figure 1B, lane 1), which corresponded to the same set of fragments protected by RNA from the hybridoma 17.2.25 (Figure 1B, lane 2). The initiation of transcription of the transfected μ gene therefore appears to occur at the same site as in the endogenous rearranged V_H 17.2.25 gene, which has been mapped to the nucleotide by Mung bean nuclease mapping (Grosschedl et al., 1985; see Figure 4B). The synthesis of transcripts derived from the modified H4 histone gene was measured

by S1 nuclease mapping using a 5′ end-labeled DNA probe obtained from the wild-type H4 gene. Hybridization of total RNA from transfected MPC11 cells to the probe and S1 nuclease treatment generated a 99 nucleotide fragment, which was protected by RNA derived from the modified H4 gene (Figure 1C).

The Ig Heavy Chain Enhancer Can Be Functionally Replaced by the Moloney-Murine Leukemia Virus Enhancer

To investigate the role of the Ig heavy chain gene enhancer (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983) in determining tissue specificity of gene expression and modulation of the transcription level during B cell differentiation, we first analyzed the effects of deleting the IgH enhancer or replacing it with a tissue nonspecific viral enhancer. Deletion of all J_H/C_μ intron DNA sequences between the Xba I sites (e.g., deletion mutant $p\mu\Delta1$; Figure 1A) abolished detectable levels of μ gene transcription in transfected MPC11 myeloma cells (Figure 2B, lane 1). The same phenotype was observed with deletion mutant $p\mu\Delta2$, which lacked the 1 kb Xba I_H DNA fragment containing the enhancer (Figure 1A; data not shown). In contrast, the 3.3 kb Xba I_H DNA fragment comprising the heavy chain gene switch-region between the enhancer and the C_μ exons could be deleted (mutant $p\mu\Delta3$; Figure 1A) without any phenotypic effect in MPC11 cells, implying that these DNA sequences are dispensable for heavy chain gene expression in myeloma cells (data not shown).

To replace the μ enhancer with a viral enhancer, we inserted each enhancer element 5′ to the promoter and thus avoided structural differences in the mRNA precursor derived from each construct. Deletion mutant $p\mu\Delta1$ was used as a recipient for the insertion of the enhancer elements. The μ enhancer residing in the 1 kb Xba I_H fragment was inserted into the Hind III site 1 kb 5′ to the promoter in either orientation (Figure 2A). Transfection of the DNA constructs, termed $p\mu\Delta1(\mu E1)$, with the μ enhancer in normal orientation, and $p\mu\Delta1(\mu E1^*)$, with the μ enhancer in reversed orientation, into myeloma MPC11 cells resulted in the accumulation of μ transcripts to a level 50% of that observed with the wild-type template (Figure 2B, lanes 2–4). The quantitation of μ gene transcription was performed by densitometric scanning of autoradiograms for transcript bands specific for μ heavy chain and H4 reference genes. The transcripts of the H4 reference gene were used to normalize the levels of specific μ mRNA (Table 1). The same number of μ transcripts was obtained from the mutant DNA constructs $p\mu\Delta1(\mu E2)$ and $p\mu\Delta1(\mu E2^*)$, which had a 700 bp Xba I/Eco RI DNA fragment comprising the IgH enhancer inserted 5′ to the promoter (Figure 2A; data not shown).

A viral enhancer was obtained from the long terminal repeat (LTR) of Moloney murine leukemia virus (M-MuLV) and inserted into the Hind III site 5′ to the V_H promoter (Figure 2A). The enhancer of M-MuLV (Mo enhancer) is functional in fibroblast cells (Laimins et al., 1982). The functional assay of the resulting constructs $p\mu\Delta1$(MoE) and $p\mu\Delta1$(MoE*) in myeloma MPC11 cells revealed that the viral enhancer in either orientation could activate transcription of the μ heavy chain gene in myeloma cells to 50% of the level obtained with the μ enhancer (Figure 2B, lanes 5 and 6). Thus, the tissue-nonspecific viral enhancer can substitute for the IgH enhancer in myeloma cells.

Table 1. Relative Transcript Level for RNA from Mutant μ Genes and Hybrid Genes in Fibroblast, Pre-B, and Myeloma Cells

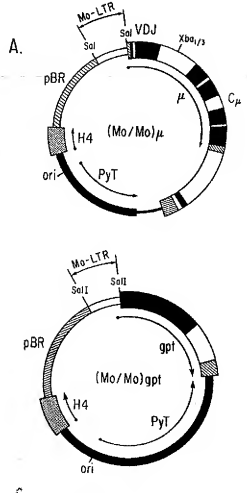
Gene Construct	mRNA	NIH/3T3	PD36	MPC11
		Relative Transcript Level		
μ (wild type)	μ	<0.01	0.4	2.0
$\mu\Delta1$	μ	<0.01	<0.01	<0.01
$\mu\Delta1(\mu E1)$	μ	<0.01	0.2	1.0*
$\mu\Delta1$(MoE)	μ	<0.01	0.2	0.5
(Mo/Mo)μ	Moμ	0.31	1.5	4.9
(Mo/Mo)gpt	Mogpt	0.52	0.27	1.0*
(μ/μ)gpt	μgpt	0.03	0.36	1.0*
(Mo/μ)gpt	μgpt	0.07	0.48	0.56
(Mo/μ^*)gpt	μgpt	0.07	0.54	0.63

The structure of the gene constructs is shown in Figures 1A, 2A, 4A, and 5A. In the first four lines, the relative transcript level (RTL) of wild-type and mutant μ genes is indicated. $\mu\Delta1(\mu E1)$ and $\mu\Delta1$(MoE) have the IgH enhancer or the M-MuLV enhancer inserted at the 5′ end of an enhancerless μ gene. In the other lines, the RTL of various hybrid genes are presented. The enhancer/promoter elements are written in parentheses. Transcripts from the hybrid genes have either 30 nucleotide M-MuLV(Mo) sequences or 57 nucleotide μ sequences attached to their 5′ ends. The RTL was determined for NIH/3T3 fibroblasts, PD36 pre-B cells, and MPC11 myeloma cells. For the determination of the RTL, various exposures of the autoradiograms were scanned densitometrically. To calculate the RTL of each gene construct, the experimental band intensity was divided by the intensity of the H4 histone reference signal. This ratio was normalized within each set of data to an arbitrarily chosen standard as value 1.
* Standard value.

The Enhancer Does Not Solely Determine Tissue Specificity of Heavy Chain Gene Transcription

To examine the transcriptional activity of the μ gene containing the IgH or the Mo enhancer in lymphoid and in nonlymphoid cell lines, we first determined whether the tissue specificity of heavy chain gene transcription is maintained in our assay. When we transfected wild-type and mutant μ genes lacking the Ig enhancer into NIH/3T3 fibroblastic cells, virtually no μ transcripts could be detected from either template, suggesting that the gene is transcriptionally inactive in fibroblastic cells (Figure 2C, lanes 1–3). From the ratio of reference and endogenous H4 transcripts, we estimated the transfection efficiency of fibroblasts to be similar to that of myeloma MPC11 cells (not shown). Because we could detect 1% of the μ mRNA signal detected in myeloma cells, we conclude that the μ gene in fibroblasts is at least 100-fold less transcriptionally active than in myeloma cells (Table 1).

The heavy chain enhancer is inactive in fibroblastic cells when tested on tissue-nonspecific transcription units (Banerji et al., 1983; Gillies et al., 1983; Grosschedl and Baltimore, unpublished). If the heavy chain enhancer were the sole determinant for tissue specificity of gene expression, it should be possible to activate the transfected heavy chain gene in fibroblast cells by providing an enhancer that is functional in these cells. Transfection of the



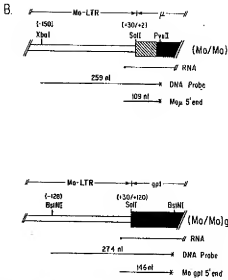


Figure 3. Intragenic μ Sequences Regulate Tissue-Specific Ig Gene Expression

(A) Structure of hybrid genes consisting of M-MuLV LTR sequences linked to either μ intragenic sequences or *gpt* gene sequences. (Top) the structure of the (Mo/Mo)μ gene construct is shown comprised of the M-MuLV enhancer and promoter fused to μ intragenic sequences that were deleted between the Xba, and Xba_3 restriction sites. (Bottom) the plasmid (Mo/Mo)*gpt* is depicted. The *gpt* gene was derived from pSV2*gpt* (Mulligan and Berg, 1980) and is comprised of *gpt* coding sequences (filled wide bar) and an SV40-derived splice site (open box) plus polyadenylation site (hatched box).

(B) Scheme of S1 nuclease mapping of transcripts from the hybrid genes. The structure of the 5' ends of the (Mo/Mo)μ gene (top) and the (Mo/Mo)*gpt* gene (bottom) are shown. Viral LTR sequences with their 5' ends terminating at +30 (relative to the viral transcription initiation site) were fused to μ sequences at +2 (relative to the μ mRNA cap site) and to *gpt* sequences at +120 (relative to the Hind III site upstream of the coding sequences, as defined in Mulligan and Berg, 1980), respectively. Restriction sites used for preparation of the 5' end-labeled, single-stranded DNA probes are indicated. The structures and the sizes of the DNA probes and protected DNA fragments are shown. Because the μ and *gpt* transcripts derived from the hybrid genes contain 30 viral nucleotides attached to their 5' ends, the hybrid transcripts are termed Moμ and Mo*gpt*, respectively.

(C) Expression of (Mo/Mo)μ and (Mo/Mo)*gpt* genes in various cell lines. Total RNA (20 μg) from cells transfected with hybrid gene constructs was analyzed by S1 nuclease mapping. The positions of the DNA fragments protected by 5' terminal sequences of the hybrid Moμ and Mo*gpt* transcripts are indicated. The slower migrating fragments represent the full-length DNA probes. In the lower gel, the S1 nuclease analysis of H4 reference gene transcripts is shown. Lane M, Hpa II-cleaved pBR322 DNA as size marker.

DNA constructs p$\mu\Delta$1(MoE) and p$\mu\Delta$1(MoE^{-}), in which the IgH enhancer had been replaced by the viral Mo enhancer, into NIH/3T3 fibroblastic cells did not, however, result in the accumulation of μ transcripts to detectable levels (Figure 2C, lanes 6 and 7). Transcription of the modified H4 gene was used to control for the transfection efficiency (Figure 2C, bottom). Because a tissue-nonspecific enhancer was not sufficient to allow heavy chain gene activation in nonlymphoid cells, sequences in the remaining part of the Ig gene must limit μ gene transcription in a tissue-specific manner.

Intragenic Sequences Contribute to the Tissue Specificity of Gene Expression

To investigate whether the promoter and/or intragenic sequences contribute to the tissue specificity of the Ig transcription unit, we separated promoter and intragenic sequences and tested them individually. For the purpose of this study, we define μ intragenic sequences as the transcribed region of the mutant $\mu\Delta1$ gene, including some downstream sequences, but lacking the IgH enhancer and most of the large V_HDJ_H/C_μ intron (Figure 1A). We examined the role of μ intragenic sequences in tissue-specific Ig gene expression by measuring the accumulation of RNA from a hybrid gene with μ intragenic sequences linked to tissue-nonspecific transcriptional control signals in myeloma and in fibroblast cells. Murine retroviral LTR sequences from position −530 to +30 (relative to the mRNA cap site), including enhancer and promoter, were fused via synthetic Sal I DNA linkers to μ intragenic sequences (Figure 3A). In this hybrid gene, termed (Mo/Mo)μ, the μ intragenic sequences were obtained by deleting all 5′-flanking sequences of the mutant $\mu\Delta1$ gene (Figure 1A) upstream of position +2 (Figures 3A and 3B). For comparison, a similar hybrid gene, termed (Mo/Mo)*gpt*, was constructed. This gene had viral LTR sequences linked to a tissue-nonspecific transcription unit consisting of the bacterial *gpt* gene and SV40-derived splice and polyadenylation sites (Figure 3A). Both hybrid genes were cloned into the short-term expression vector and transfected into MPC11 myeloma and NIH/3T3 fibroblast cells. Synthesis of specific transcripts was assayed by S1 nuclease mapping. For both genes, transcripts began at the expected site within the LTR and contained 30 viral nucleotides attached to their 5′ ends (Figure 3B). Transcripts from the hybrid (Mo/Mo)μ gene were detected in both MPC11 and NIH/3T3 cells (Figure 3C, lanes 2 and 3). We calculated the transcription level of this hybrid gene in both cell types using transcription of the H4 reference gene for normalization (Table 1). MPC11 cells contained 16-fold more Moμ RNA than did NIH/3T3 cells. In comparison, transcripts of the (Mo/Mo)*gpt* gene were detected in myeloma cells at a 2-fold higher level than in fibroblasts (Figure 3C, lanes 4 and 6; Table 1). Therefore, intragenic sequences allowed for an 8-fold enhanced accumulation of transcripts from the (Mo/Mo)μ gene in myeloma cells, as compared with fibroblastic cells. This result suggests that μ intragenic sequences contribute significantly to the tissue specificity of μ gene expression.

Tissue- and Cell-Type-Specificity of V_H Promoter Sequences

Replacement of the Ig enhancer and 5′-flanking sequences of the μ gene with viral transcription signals led to a partial activation of this gene in fibroblastic cells (Figure 3C, lane 2). In contrast, substitution of the IgH enhancer alone with a tissue-nonspecific enhancer did not allow for any detectable transcription in nonlymphoid cells (Figure 2C, lanes 6 and 7). These data suggest that 5′-flanking μ gene sequences play a crucial role in determining tissue specificity of μ gene transcription. To examine whether V_H promoter sequences are important for tissue-

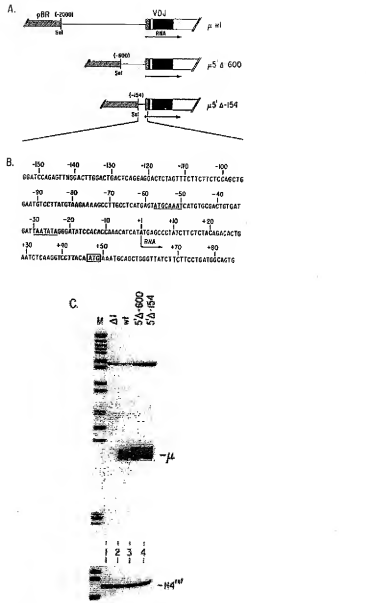


Figure 4. Delimitation of V_H Promoter Sequences

(A) Structure of 5′ deletion mutants. The 5′-flanking sequences of the rearranged μ gene are represented as thin lines. Noncoding V_H-derived sequences are drawn as a hatched area, exons as black, introns as open. The nucleotide positions of the boundaries of the 5′-flanking sequences are indicated. (B) Nucleotide sequence of the rearranged V_HDJ_H region between −154 and +85 relative to the transcription initiation site. The mRNA cap site (Grosschedl et al., 1985) is indicated. The TATA consensus sequence and an octanucleotide sequence conserved among all known V regions (Parslow et al., 1984), are underlined. The initiation codon for translation is boxed. (C) Expression of 5′ deletion mutants in MPC11 myeloma cells. MPC11 cells were transfected with wild-type and mutant μ genes. Total RNA was isolated and analyzed for expression of the μ genes and the H4 reference genes by S1 nuclease mapping as described in Figure 1. The positions of protected μ and $H4^{rat}$ fragments are indicated. The upper band corresponds to the full-length DNA probe.

specific gene expression, we first delimited the 5′ boundary of the functional promoter by constructing two mutant μ genes lacking portions of 5′-flanking sequences. One deletion mutant, $p\mu5'\Delta$-600, was generated by fusing the Hinc II site at nucleotide position −600 from the site of transcription initiation onto the pBR322 vector sequences (Figure 4A). Another mutant, $p\mu5'\Delta$-154 was obtained by deleting all sequences between the Sal I site of the vector and the Bam HI site at position −154 of the μ gene. Both mutants contained the Ig enhancer in its normal position in the large intron between the V_HDJ_H and the C_μ region. The 5′ deletion mutants of the μ gene were transfected into MPC11 myeloma cells, total RNA was isolated and analyzed for specific μ and H4 transcripts. As a positive control, the wild-type μ gene was used. Both mutant templates directed μ gene transcription at a 2-fold higher level than did the wild-type DNA, indicating that the 5′-flanking sequences upstream of position −154 can be removed without deleterious effect on μ gene transcription (Figure 4C, *lanes* 2–4). The 5′ boundary of the V_H promoter therefore mapped downstream of nucleotide position −154.

To examine the role of the V_H promoter for tissue- and cell-stage-specificity of Ig gene transcription, we constructed a hybrid gene by joining V_H promoter sequences to *gpt* gene coding sequences. Two promoter fragments, with their 5′ boundaries at nucleotide position −600 and −154, respectively, and their 3′ boundaries at position +57, were isolated from the rearranged μ gene. A Pvu II restriction site at position +57 was chosen as the 3′ boundary of the promoter to retain DNA sequences around the initiation site of transcription. Because the V_H promoter of the rearranged heavy chain gene is virtually inactive without an enhancer, we ligated either the IgH enhancer or the tissue-nonspecific M-MuLV enhancer to the 5′ end of the V_H promoter fragments. The enhancer/promoter regulatory elements were fused to the bacterial *gpt* gene containing SV40 splice and polyadenylation sites and were cloned into the short-term expression vector (Figure 5A). The resulting hybrid genes, $(\mu_H)gpt$ and $(Mo\mu_H)gpt$, have the μ IgH enhancer and the Mo enhancer, respectively, linked to the long V_H promoter fragment. The $(Mo\mu')gpt$ gene construct contained the Mo enhancer and the short V_H promoter fragment. The hybrid genes were transfected into MPC11 myeloma cells, and transcription was assayed by S1 nuclease mapping (Figure 5B). Initiation of the *gpt* transcripts occurred at the normal initiation site within the V_H promoter fragments. Therefore, the *gpt* transcripts contained 57 nucleotides of the rearranged μ gene attached to their 5′ end. Few μgpt transcripts were synthesized from any of the hybrid gene (Figure 5C, lanes 4–6). The transcription level of the $(\mu_H)gpt$ gene was 2-fold higher than that of the $(Mo\mu_H)gpt$ gene construct, indicating the slightly lower activity of the Moloney virus enhancer (cf. Figure 2B). Adjusting for the transfection efficiency indicated by the H4 reference gene transcription, the $(Mo\mu')gpt$ gene was as active as the $(Mo\mu_H)gpt$ gene (Table 1). The relatively low transcriptional activity of these hybrid genes in myeloma cells can be at least partially explained by the contribution of μ intragenic sequences to gene expression in these cells. The μ transcripts derived from the hybrid $(Mo/Mo)\mu$ gene accumulate in myeloma cells at a 5-fold higher level than do *gpt* transcripts obtained from the $(Mo/Mo)gpt$ gene (Figure 3C, lanes 3 and 6; Table 1).

To determine the activity of the V_H promoter region in fibroblastic cells, we tested the hybrid genes in NIH/3T3 cells. A very small number of μgpt transcripts could be detected in total RNA from NIH/3T3 cells transfected with the $(\mu_H)gpt$ gene (Figure 5C, lane 7). Replacement of the IgH enhancer with the tissue-nonspecific Moloney virus enhancer in the gene construct $(Mo\mu_H)gpt$ increased the *gpt* transcription level only 2-fold (Figure 5C, lane 8; Table 1). Using the H4 reference gene expression for normalization, we calculated the transcriptional activity of the $(Mo\mu_H)gpt$ gene to be about 10-fold higher in myeloma cells than in fibroblast cells (Table 1). These results imply that the V_H promoter region can confer tissue specificity to the bacterial *gpt* gene, even in the presence of a viral enhancer. The phenotype of the $(Mo\mu')gpt$ gene was identical with that of the $(Mo\mu_H)gpt$ gene construct (high expression in lymphoid cells, low expression in nonlymphoid cells; Figure 5C, lanes 5, 6, 8, and 9). Thus, the V_H promoter region between −154 and +57 appears to contain DNA sequences required for tissue-specific transcription.

Regulation of Ig Gene Expression during B Cell Differentiation

Although between pre-B and plasma cell stages the level of accumulated heavy chain transcripts increases 30- to 100-fold (Perry and Kelley, 1979), the rates of Ig heavy chain transcription between these cell types differ only by a factor of 2–5 (Mather et al., 1984). To examine whether Ig gene transcription depends on enhancer activity at the early stage of B lymphoid cell differentiation, we assayed transcription of wild-type and mutated μ gene constructs in pre-B and in B cells.

As representative pre-B cells, we used the Abelson virus-transformed cell line PD36 as recipient for DNA transfection experiments. These cells have their endogenous μ gene locus rearranged and are undergoing κ gene rearrangement (Lewis et al., 1982). Transfection of the wild-type μ gene resulted in the accumulation of specific μ transcripts at a level 5 times less than in MPC11 cells (Figure 6A, lane 1, Table 1). Thus, in the transient expression assay, the transfected Ig gene is only 5 times less active in pre-B cells than in myeloma cells. To test the enhancer requirement for μ gene transcription in PD36 cells, we introduced the Ig enhancerless constructs $p\Delta1$ and $p\Delta2$ into these cells. No specific μ transcripts could be detected by S1 nuclease mapping (Figure 6A, lanes 2 and 3), implying that the enhancer is required for μ gene transcription at this early stage of the B cell lineage. Gene transcription could be restored to 50% of the wild-type level when the IgH enhancer or the Mo enhancer was inserted 5′ to the V_H promoter (Figure 6A, lanes 5–8). As representative mature B cells we transfected WEHI 279.1 cells (Warner et al., 1979) with the various μ gene constructs (Figure 6B). As in pre-B cells, an enhancer was required for transcription, but no differential activity between the Mo and IgH enhancers was evident.

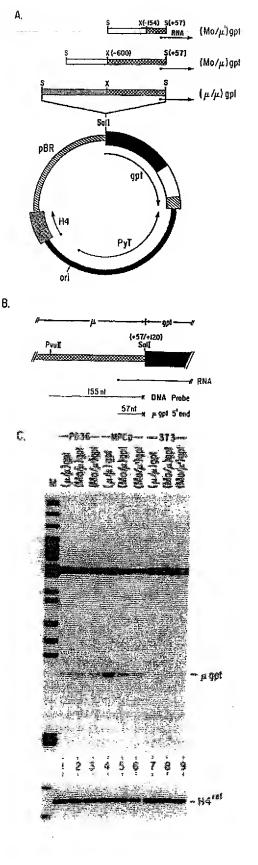


Figure 5. Tissue-Specificity of V_H Promoter Sequences

(A) Schematic representation of hybrid gene constructs containing M-MuLV or IgH enhancer, V_H promoter and *gpt* coding sequences. The various enhancer/promoter fragments used for fusion to *gpt* gene sequences are shown in linear form above the circular map. V_H promoter fragments are drawn as crosshatched bars with their boundaries given in nucleotides relative to the initiation site of RNA synthesis (arrow). The μ IgH enhancer fragment is shown as a dotted bar and consists of the 700 bp Xba I/Eco RI DNA fragment (see Figure 2A) with the Eco RI site converted to a Sal I site. The viral Mo enhancer is represented by an open bar. Restriction sites are abbreviated as S for Sal I and X for Xba I. The structure of the *gpt* gene is identical with that shown in Figure 3A.

(B) Scheme of S1 nuclease mapping of RNA from hybrid genes. The structure of the 5' end of the hybrid genes is shown. The V_H promoter region is drawn as a crosshatched bar, *gpt* coding sequences as a black box. The 3' boundary of the V_H promoter fragment at nucleotide position +57, relative to the transcription initiation site, was ligated via a Sal I DNA linker to *gpt* sequences at +120 (relative to the Hind III site upstream of the coding sequences as defined in Mulligan and Berg, 1980). The structures and sizes of the DNA probe and the protected fragment are indicated.

(C) Analysis of RNA from hybrid genes in various cell types. Total RNA (50 μg) from various cell lines transfected with (μ/μ)*gpt*, (Mo/μ)*gpt*, and (Mo/μ')*gpt* was analyzed by S1 nuclease mapping for specific *μgpt* and H4^{ref} transcripts. The position of the DNA fragment protected by 5' terminal *μgpt* RNA sequences is indicated as *μgpt*. The slower migrating band represents the full-length DNA probe. Cell lines and constructs are indicated above each lane.

Using the other gene constructs previously examined in MPC11 cells, we studied the sequence elements required for Ig gene expression in PD36 cells. The (Mo/μ)*gpt* and (Mo/μ')*gpt* genes were expressed at the same level in PD36 and MPC11 cells (Figure 5C, lanes 2, 3, 5 and 6; Table 1), suggesting that the V_H promoter linked to the Mo enhancer is as active in pre-B cells as in terminally differentiated cells. Analysis of the transcriptional activity of the (μ/μ)*gpt* gene in PD36 cells revealed that transcripts from this gene accumulate at a level 3 times lower than in MPC11 cells (Figure 5C, lanes 1 and 4; Table 1). To test the influence of μ intragenic sequences on gene expression in pre-B cells, we measured the levels of transcripts derived from the (Mo/Mo)*gpt* and $(Mo/Mo)\mu$ genes in PD36 cells (Figure 3C, lanes 1 and 5). Transcripts from the $(Mo/Mo)\mu$ gene accumulated at a 5-fold higher level, as was found in MPC11 cells (Table 1), implying that μ intragenic sequences increase mRNA accumulation in both lymphoid cell types. Furthermore, taking into account the 2-fold lower transcriptional activity of the (Mo/Mo)*gpt* gene in PD36 cells, as compared with NIH/3T3 cells, the $(Mo/Mo)\mu$ gene appears to be 10-fold more active in pre-B cells than in fibroblastic cells (Table 1). Thus, we see no major difference in the requirement for regulatory Ig gene sequences in PD36 and in MPC11 cells.

Discussion

Regulation of Tissue-Specific Ig Gene Expression

Immunoglobulin heavy chain genes are transcriptionally active only in lymphoid cells, and their level of expression is regulated within this cell lineage (Perry and Kelley, 1979). From experiments involving transfection of rearranged genes into cultured cells (Gillies et al., 1983; Neuberger, 1983) and from germline gene transfer experi-

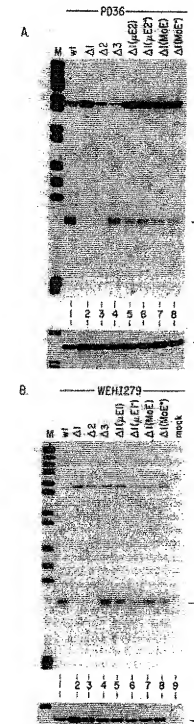


Figure 6. Transcriptional Activity of the Wild-Type and Mutant μ Gene Constructs in Pre-B Cells (P036) and in Mature B Cells (WEHI 279.1)

(A) Analysis of 5′ ends of specific μ transcripts in transfected P036 cells by S1 nuclease mapping. Total RNA (60 μg) was hybridized to the μ DNA probe and digested with S1 nuclease. For the analysis of $H4^{ref}$ transcripts, 5 μg total RNA was used. (B) S1 nuclease analysis of specific μ and H4 transcripts in total RNA from transfected WEHI 279.1 cells.

ments (Grosschedl et al., 1984), it has become clear that the elements controlling this regulation are located near the gene and are active in *cis*. We have studied the genetic basis for the tissue specificity of Ig heavy chain gene expression by dissecting the transcription unit into various elements, each of which contains regulatory information.

Virtually no expression of a transfected wild-type μ gene has been detected in fibroblast cells. Because transfected heavy chain genes require an enhancer in order to be expressed at high levels in lymphoid cells (Gillies et al., 1983; Neuberger, 1983; this paper), the lack of Ig gene transcription in fibroblast cells could be formally explained by an inactivity of the enhancer. Indeed, the heavy chain gene enhancer is inoperative in fibroblastic cells, because it can activate heterologous genes in lymphoid cells but not in fibroblastic cells (Banerji et al., 1983; Gillies et al., 1983; Grosschedl and Baltimore, unpublished). Expression of the μ heavy chain gene, however, remained lymphoid-specific when the Ig enhancer was replaced by the tissue-nonspecific enhancer from M-MuLV, implying that the IgH enhancer is neither the only, nor necessarily the dominant, regulatory element for the control of tissue specificity of Ig gene expression. Our experiments demonstrated that two additional regulatory sequences, the V_H promoter region and the gene itself, also regulate the tissue specificity of expression.

The Role of the Ig Enhancer for Gene Expression

In myeloma cells, transcription of a transfected rearranged Ig heavy chain gene was shown to be dependent on the presence of an enhancer (Gillies et al., 1983; Neuberger, 1983; Figure 2B). We addressed the question of whether the enhancer is required for gene expression at earlier stages of cell differentiation by introducing the wild-type μ gene and μ gene constructs without an enhancer or with the Ig enhancer replaced by a viral enhancer into pre-B, mature B, and myeloma cell lines. No specific μ transcripts were evident from the enhancerless μ gene in any of the cells. Transcription of the μ gene could be stimulated by the Ig enhancer at all three major stages of the B cell lineage, implying that the Ig enhancer is functional throughout B cell differentiation. Our results are in agreement with the detection of sequence-specific interactions of the IgH enhancer region with cellular factors at all three stages of B cell differentiation (Ephrussi et al., 1985). Therefore, the heavy chain enhancer appears to be tissue-specific but not cell-stage-specific. In this respect the Ig enhancer differs from the E_β gene enhancer, which was shown to be functional in mature B cells but inactive in myeloma cells (Gillies et al., 1984).

In some lymphoid T cell lines the Ig heavy chain locus is transcriptionally active, and "sterile" μ transcripts are generated (Kemp et al., 1980; Alt et al., 1982). These sterile μ transcripts initiate near the J_H gene segments (Nelson et al., 1983). We tested the activity of the Ig enhancer after transfection of μ gene constructs with and without enhancer into EL4 cells, a line that contains the "sterile" μ transcripts, and demonstrated that the Ig enhancer could stimulate transcription in these cells (unpublished results).

This observation suggests that the enhancer may be involved in the developmental activation of the heavy chain locus at a very early stage of lymphoid cell differentiation.

Recently the functional importance of the Ig heavy chain enhancer for high level Ig gene expression has been brought into question by the observation that spontaneous deletions of heavy chain gene intron sequences, including the enhancer that occurred during growth of hybridoma cells, did not affect the level of Ig gene expression (Wabl and Burrows, 1984; Klein et al., 1984). A possible explanation for this observation is that the Ig enhancer is required at the initial stage of gene activation but is dispensable for maintenance of transcription. Our experiments do not rule out such a mechanism for enhancer activity because, by transfecting DNA into various cultured cells, we might mimic, in each case, the initial stage of gene activation. The results that the IgH enhancer can stimulate transcription at all major stages of the B cell lineage, however, indicated that the permissive conditions for Ig enhancer activity are present throughout B cell differentiation. Further support for the constitutive activity of the IgH enhancer in the lymphoid B cell lineage comes from the examination of the enhancer DNA sequences for physical interaction with *trans*-acting factors (Ephrussi et al., 1985). A persistent pattern of protection of specific nucleotides against methylation by dimethylsulphate suggested that a protein remains bound to the DNA during cellular differentiation.

The V_H Promoter Region Controls Tissue-Specific Gene Expression

We have shown that the V_H promoter region between nucleotide positions −154 and +57 can confer tissue specificity to the bacterial *gpt* gene. Transcripts of *gpt* initiating at V_H promoter sequences accumulate in myeloma cells at a 10-fold higher level than in fibroblast cells. The V_H promoter region can direct lymphoid-specific transcription even in the presence of a viral enhancer, suggesting that it acts as a dominant genetic element that can mask an active enhancer in nonlymphoid cells.

The precise location of the tissue-specific regulatory sequence within the V_H promoter region between −154 and +57 has not yet been identified. Transcripts of the hybrid (Molμ)*gpt* gene initiating at V_H promoter sequences contain the 57 terminal nucleotides of μ mRNA at their 5′ end. Although these μ sequences might be involved in the differential accumulation of such *gpt* transcripts in lymphoid versus nonlymphoid cells, a likely candidate for a tissue-specific control element is the octanucleotide ATGCAAAT at position −50. DNA sequence comparison revealed that this octanucleotide is highly conserved in sequence and topology among all known variable regions of immunoglobulin genes (Parslow et al., 1984; Falkner and Zachau, 1984). A short DNA fragment that contains the inverted form of this conserved sequence appears to be possibly crucial, by functional tests, for κ gene transcription in myeloma cells (Falkner and Zachau, 1984; Bergman et al., 1984). Interestingly, the octanucleotide sequence is also present in the heavy chain enhancer (Falkner and Zachau, 1984), which might indicate that this sequence could be involved in conferring tissue specificity in both promoter and enhancer elements. Such a mechanism would allow for the tissue specificity of multiple regulatory elements by one *trans*-acting factor. The occurrence of multiple binding sites has been described for the glucocorticoid receptor in the mouse mammary tumor virus genome (Scheiderelt et al., 1983; Payvar et al., 1983).

Intragenic Sequences Contribute to Tissue-Specific Gene Expression

We have demonstrated that μ heavy chain gene transcripts initiating at tissue-nonspecific viral transcriptional control elements accumulate in lymphoid and in nonlymphoid cells at different levels. Thus it appears that μ intragenic sequences are involved in the enhanced accumulation of their transcripts in lymphoid cells. Several mechanisms can be considered to account for this tissue-specific regulation of μ transcript levels by intragenic sequences. First, it is possible that sequences within the rearranged μ gene, which lacks most of the VDJ/C_μ intron sequences, regulate the rate of transcription initiation. Intragenic sequences were demonstrated to control the transcription of genes transcribed by RNA polymerase III (Bogenhagen et al., 1980; Sakonju et al., 1980; Galli et al., 1981). Second, splicing of pre-mRNA might be controlled in a tissue-specific fashion, thus leading to different amounts of mature stable RNA in different cell types. An example for this kind of regulation might be the troponin T gene; the differential splicing of its pre-mRNA is most likely regulated by intragenic sequences (Medford et al., 1984). A third mechanism would be a regulation of the transcript level by differential stability of the mRNA, as shown for some liver-specific mRNAs (Jefferson et al., 1984).

The functional importance of intragenic sequences for gene expression has been recently demonstrated for other genes transcribed by RNA polymerase II. Expression of the chicken *tk* gene in differentiating muscle cells is regulated by gene-internal sequences (Merrill et al., 1984). The stimulation of β globin expression in murine erythroleukemia cells by dimethyl sulphoxide and in HeLa cells by enhancers was also shown to be determined by intragenic sequences (Charnay et al., 1984; Wright et al., 1984; Treisman et al., 1983). The mechanism by which these intragenic sequences regulate gene expression has yet to be established for any gene.

Our evidence therefore implicates three elements in controlling the tissue-specific expression of an IgH chain. The enhancer acts only in lymphoid cells but can stimulate gene expression at all stages of lymphoid cell development. The promoter provides for 10-fold greater expression in lymphoid versus nonlymphoid cells but is not differentially active at different stages of B lymphoid cell development. Intragenic sequences provide for 8-fold greater synthesis in lymphoid cells than in fibroblasts and may also be the site for regulating cell-stage-specific differences in mRNA accumulation. Because most of the cell-stage-specific regulation of the μ transcript level is posttranscriptional (Mather et al., 1984), intragenic se-

quences may regulate pre-mRNA splicing, RNA transport, or mRNA stability.

Cell-Stage-Specific Ig Gene Regulation during B Cell Differentiation

Measurements of the level of cytoplasmic μ mRNA derived from endogenous Ig genes in pre-B and myeloma cells suggested a 30- to 100-fold greater accumulation of μ transcripts in myeloma cells (Perry and Kelley, 1979). Cytoplasmic μ mRNA from stably transfected pre-B and myeloma cells were found to accumulate at a 20-fold higher rate in myeloma cells (Neuberger, 1983). In the short-term transfection assay, we observed only a 5-fold difference in the μ transcript level between pre-B and myeloma cells. We cannot account for this apparent discrepancy between in vivo data and data on transfected cells but two reasons might be considered. There is evidence that the ratio of nuclear RNA to cytoplasmic RNA is 50-fold higher in pre-B cells, as compared with myeloma cells (Perry and Kelley, 1979). Because we used total RNA for the analysis of the μ transcript level, rather than cytoplasmic RNA, the quantitative difference of μ transcripts between the two cell stages might be diminished. Another reason might be a consequence of the experimental system itself. We assayed total RNA from transfected cells two days after transfection. The early region of polyoma allows for the amplification of the DNA template, and the majority of the transcripts might be synthesized relatively late in the incubation period. Hence, a differential posttranscriptional regulation between pre-B and myeloma cells may not be seen to its full extent. Indeed most of the quantitative difference in the transcript level between pre-B and myeloma cells was found to be caused by posttranscriptional regulation (Mather et al., 1984).

Whatever the explanation for the lack of a quantitative difference in μ mRNA accumulation between pre-B cells and plasma cells, the various gene constructs show qualitatively similar behavior in the two cell types. Therefore, μ gene expression at all stages of B lymphoid cell differentiation is probably regulated by the same set of DNA elements located in the enhancer, promoter, and intragenic segments.

Experimental Procedures

Construction of Plasmids

The DNA construct referred to in this paper as pμ is the same as the one described in Grosschedl et al. (1984) with the exception that the early region of polyoma virus (position 4631 to 2962; Griffin et al., 1980) has been inserted between the immunoglobulin gene and histone H4 gene. To generate the enhancer insertion mutations, a subclone of the rearranged μ gene was constructed. The Sal I-Xho I DNA fragment of p$\mu\Delta$1 (see Figure 1A) harboring the VDJ region was cloned into a Sal I-Xma I-cleaved pUC13 vector that had no Hind III site. The resulting subclone was linearized with Hind III 1 kb upstream of the V_H transcription initiation site, and various DNA fragments carrying IgH or M-MuLV enhancer elements were linked with Hind III linkers and inserted into the Hind III site.

For the construction of the hybrid (Mo/Mo)μ gene, the plasmid p$\mu\Delta$1 was partially cleaved with Nde I, blunt-ended and ligated with synthetic Sal I linkers. The plasmid that had the Nde I site at position +2 (relative to the μ mRNA cap site) converted to a Sal I site, was cleaved with Sal I and recircularized under dilute conditions thus deleting all μ 5'-flanking sequences. A Cla I/Sma I DNA fragment containing LTR sequences of M-MuLV was ligated to Sal I linkers and inserted into the Sal I site of this $\mu\Delta$1 gene construct lacking all μ 5'-flanking sequences.

The hybrid *gpt* genes were constructed by the following scheme: pSV2*gpt* (Mulligan and Berg, 1980) was cleaved with Bgl II at position +120 of the *gpt* gene (counting the nucleotides from the Hind III site) and with Bam HI downstream of the SV40-derived splice and polyadenylation site. The DNA fragment containing the *gpt* gene was blunt-ended and ligated to Sal I linkers. The Sal-linked *gpt* DNA fragment was then ligated with Sal I-Xho I-cleaved vector DNA containing the H4 gene and the polyoma early region. The plasmid with a unique Sal I site at the 5' end of the *gpt* gene was used for further constructions. To prepare the large V_H promoter fragment, a Hind III-Stu I DNA fragment containing the 5' half of the $V_{17.2.25}$ gene and 5'-flanking sequences was isolated from the rearranged μ gene. The DNA fragment was partially cleaved with Pvu II and ligated with Sal I linkers. After recutting with Sal I, the V_H promoter DNA fragment, with the Pvu II site at position +57 (relative to the mRNA cap site) converted to a Sal I site, was isolated. This DNA fragment was cleaved with Hinc II at position −600 and was ligated with Xba I linkers. After recutting the fragment with Xba I, the Xba I-Sal I-linked V_H promoter fragment was purified by gel electrophoresis and was cloned into pUC13. The small V_H promoter fragment was obtained by converting the Bam HI site at position −154 within the large V_H promoter fragment into an Xba I site. The Xba I-Sal I-linked promoter fragments were ligated with Xba I-Sal I-linked IgH- or Moloney virus-enhancer fragments and cloned into the Sal I site of the *gpt* gene plasmid.

Cell Culture and DNA Transfection

MPC11 myeloma cells were grown in DME supplemented with 5% calf serum and 5% fetal calf serum. NIH/3T3 cells were grown in DME containing 10% calf serum. Other lymphoid cells were grown in RPMI containing 10% fetal calf serum and 50 μM 2-Mercaptoethanol.

For transfections, 10^7 lymphoid cells were washed with TS (8 g/l NaCl, 0.38 g/l KCl, 0.1 g/l $Na_2HPO_4 \cdot 7H_2O$, 3.0 g/l Tris, 0.1 g/l $MgCl_2$, 0.1 g/l $CaCl_2$), pH 7.4 (Sompayrac and Danna, 1981), and were resuspended in 1.5 ml TS containing DNA at 2 μg/ml and DEAE-dextran (Pharmacia; M_r 5 × 10^5) at 0.5 mg/ml. The mixture was kept for 20 min at room temperature. Tissue culture medium (15 ml) containing 0.1 mM Chloroquine diphosphate (Sigma; Luthman and Magnusson, 1983) was added, and after 60 min at 37°C the Chloroquine-medium was replaced with normal tissue culture medium. The cells were harvested after 42-48 hr incubation, and total RNA was isolated and purified as described (Chirgwin et al., 1979; Glisin et al., 1974).

S1 Nuclease Analysis

From 5 to 50 μg total RNA was hybridized with single-stranded ^{32}P 5' end-labeled DNA fragments at 2.5 μg/μl as described (Hentschel et al., 1980). tRNA was used to adjust the amount of total RNA to at least 25 μg. The hybridization temperatures were 38°C for the μ DNA probe and 50°C for the H4, Mo*gpt* and Moμ DNA probes. The hybrids were digested with 50 units of S1 nuclease (PL Biochemicals) at 37°C for 1 hr. The protected DNA fragments were analyzed by electrophoresis through 8% polyacrylamide-urea gels.

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Multiple Nuclear Factors Interact with the Immunoglobulin Enhancer Sequences

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Summary

To characterize proteins that bind to the immunoglobulin (Ig) heavy chain and the κ light chain enhancers, an electrophoretic mobility shift assay with end-labeled DNA fragments was used. Three binding proteins have been found. One is NF-A, a factor found in all tested cell types that binds to the octamer sequence found upstream of all Ig variable region gene segments and to the same octamer in the heavy chain enhancer. The second, also ubiquitous, protein binds to a sequence in both the heavy chain and the κ enhancers that was previously shown to be protected from methylation in vivo. Other closely related sites do not compete for this binding, implying a restriction enzyme–like binding specificity. The third protein binds to a sequence in the κ enhancer (and to an identical sequence in the SV40 enhancer) and is restricted in its occurrence to B cells.

Introduction

Immunoglobulin (Ig) gene expression is governed by three types of tissue-specific regulatory sequences (Grosschedl and Baltimore, 1985)—the promoter (Falkner and Zachau, 1984; Bergman et al., 1984; Mason et al., 1985; Gopal et al., 1985; Foster et al., 1985; Picard and Schaffner, 1985), the enhancer (Gillies et al., 1983; Banerji et al., 1983; Neuberger, 1983; Mercola et al., 1983; Queen and Baltimore, 1983; Queen and Stafford, 1984; Picard and Schaffner, 1984), and, at least in the case of the μ heavy chain gene, by an intragenic sequence as well (Grosschedl and Baltimore, 1985). Within the upstream promoter region, sequence comparisons (Parslow et al., 1984) followed by deletion analysis have indicated the importance of an octameric motif (ATTTGCAT), located at a characteristic distance upstream of all sequenced variable region genes (Falkner and Zachau, 1984; Bergman et al., 1984; Mason et al., 1985). We have recently reported the detection of a nuclear factor that interacts with this sequence (Singh et al., 1986). Enhancers were defined in viruses as regulatory sequences that can potentiate transcription from a variety of promoters in a distance- and orientation-independent manner, but the Ig enhancers were the first found to be tissue-specific as well. It has been assumed that the action of these *cis* regulatory elements must be mediated by *trans*-acting factors, and there is now some experimental evidence to support this conjecture. Schöler and Gruss (1984) and Mercola et al. (1985) have carried out in vivo competition experiments which imply that the presumed factor can be functionally titrated by cotransfection of increasing amounts of enhancer sequences. Also, Ephrussi et al. (1985) and Church et al. (1985) have obtained footprints of a putative tissue-specific factor on the heavy chain enhancer in living cells and nuclei. Recently, a substantial advance has also been made by the development of enhancer-dependent in vitro transcription systems (Sassone-Corsi et al., 1984; Wildeman et al., 1984; Sergeant et al., 1984; Schöler and Gruss, 1985). Competition experiments carried out in vitro (Schöler and Gruss, 1985; Sassone-Corsi et al., 1985) have further indicated that enhancer function may be mediated by *trans*-acting factors. To understand the mechanisms of enhancer function and their role in the activation of tissue-specific genes, we have searched for the presence of such factors in nuclear extracts.

We report here interaction of factors with Ig μ and κ enhancer sequences as detected by an electrophoretic mobility shift assay (Fried and Crothers, 1981; Garner and Revzin, 1981). This technique, based on the fact that nucleoprotein complexes are resolved from uncomplexed DNA by electrophoresis through polyacrylamide gels in low ionic strength buffers, has been elegantly used for the kinetic and equilibrium analysis of a number of prokaryotic DNA binding proteins (Hendrickson and Schliel, 1984, 1985; Fried and Crothers, 1984a, 1984b; Bushman et al., 1985). More recently it has been used to detect and purify eukaryotic proteins that were believed to recognize specific DNA sequences (Strauss and Varshavsky, 1984; Piette et al., 1985; Carthew et al., 1985; Levinger, 1985; Singh et al., 1986). Because a functional enhancer may consist of multiple protein-binding DNA segments, we have dissected the enhancer into small fragments retaining only one or two binding sites for sequence-specific proteins. In this way, at least three different Ig enhancer–binding proteins have been identified.

Results

The fully functional μ enhancer is included in a 700 bp XbaI–EcoRI fragment from the intron between J_H and C_μ. This fragment can be further subdivided into a 400 bp XbaI–PstI fragment (μ400) and a 300 bp PvuII–EcoRI fragment (μ300). Transient transfection assays have shown that 30%–50% of the tissue-specific enhancer activity is retained in μ300, whereas there is no detectable activity in μ400 (Grosschedl and Baltimore, 1985). We have used an electrophoretic mobility shift assay to investigate protein factors that interact with the μ enhancer. In outline, the assay involves incubating end-labeled, specific DNA fragments plus unlabeled, nonspecific DNA for 30 min at room temperature with nuclear extracts made from tissue culture cells (Dignam et al., 1983). Protein–DNA complexes are then separated from free DNA by electrophoresis through a low ionic strength polyacrylamide gel and

visualized by autoradiography. When the functional 300 bp enhancer fragment (μ300) was used in such an assay, a DNA–protein complex migrating more slowly than free DNA was observed with extracts derived from the human B lymphoma cell line EW (Figure 1B, lanes 1 and 2; the complex is indicated by the arrow). To show that this new band represented a specific complex, we carried out binding reactions in the presence of varying amounts of nonradioactive competitor fragments (Figure 1B, lanes 3–11). When μ300 was added as the competitor fragment (Figure 1B, lanes 3–5), the complex band was completely lost with 200 ng of competitor (lane 5), whereas the adjacent μ400 fragment (lanes 6–8) or the 450 bp fragment containing the κ light chain enhancer (lanes 9–11) yielded, at most, minor competition even at the highest concentrations used. The slight increase of the specific complex caused by the κ enhancer fragment (Figure 1B, compare lanes 9 and 2) could be due to its binding of factors common to both enhancers (described below), thus leaving more of the labeled fragment available to bind to a μ-specific factor.

Localization of Heavy Chain Enhancer Binding

To define the complex detected with μ300 more precisely, we further dissected this fragment by digestion with AluI, HinfI, and DdeI, generating a number of 50–70 bp fragments called μ50, $(\mu 60)_2$ (a mixture of μ60-1 and μ60-2), and μ70 (Figure 2A). Binding reactions were carried out with each of these fragments using EW nuclear extracts in the presence of increasing amounts of the nonspecific competitor poly d(IC) (Figure 2B). Fragment μ50 formed a major complex band (Figure 2B, lanes 2–4) that was barely decreased even in the presence of 3.6 μg of poly d(IC) (lane 4). The mixture of the two 60 bp fragments did not yield a discrete complex band (Figure 2B, lanes 6–8). Finally the μ70 fragment gave three faint, but discrete, nucleoprotein complex bands (Figure 2B, lane 10); the lower one was again barely affected by 3.6 μg of nonspecific carrier poly d(IC) (lane 12). The complex generated with μ50 was specifically competed away by inclusion of 50 ng of μ300 (of which μ50 is a part) (Figure 2C, compare lanes 3 and 2) or a κ promoter fragment (lane 7) in the binding reaction but not by inclusion of corresponding amounts of μ400 (lane 4), of the SV40 enhancer (lane 5), or of a fragment containing the κ enhancer (lane 6). This result implies that the μ50 complex is generated by interaction of the DNA with a previously described factor, NF-A (previously referred to as IgNF-A; Singh et al., 1985), that recognizes a conserved octanucleotide, ATTTGCAT, found both in the promoters of all sequenced immunoglobulin genes and within this subfragment of the heavy chain enhancer. We shall refer to this motif as the O sequence.

The complex observed with μ70 was specifically competed away by only the μ300 fragment (Figure 2D, compare lanes 3 and 2) and to some extent by the κ enhancer (data not shown), but was not at all competed away by either the Moloney murine leukemia virus enhancer (data not shown), the SV40 enhancer (data not shown), or the μ400 fragment (compare lanes 4 and 2). Furthermore,

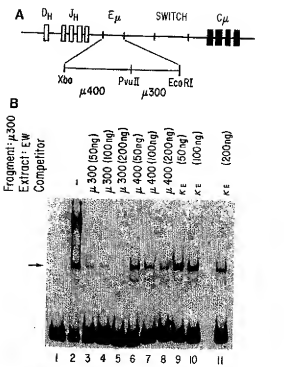


Figure 1. Factor Binding to the μ Enhancer

(A) Schematic representation of the germ line immunoglobulin heavy chain locus. The μ enhancer (E_μ) has been localized to a 700 bp XbaI–EcoRI fragment from within the J_H–C_μ intron (Grosschedl and Baltimore, 1985). When further dissected by cutting at the PvuII site, 30%–50% of the enhancer function is recovered in the 300 bp PvuII–EcoRI fragment (μ300), whereas the XbaI–PvuII fragment (μ400) does not carry any detectable enhancer function by transient transfection assay (Grosschedl and Baltimore, 1985).

(B) Electrophoretic mobility shift assay of μ300. End-labeled μ300 (10,000 cpm, 0.5 ng) was incubated with 8 μg of a nuclear extract derived from the human B lymphoma EW in the presence of 3.6 μg of poly d(IC) and various amounts of nonradioactive competitor DNA fragments as noted, followed by electrophoresis through a low ionic strength polyacrylamide gel. The specific nucleoprotein complex is indicated by the arrow. Lane 1, free DNA fragment. Lane 2, binding reaction in the absence of competitor DNA. Lanes 3, 4, 5 are 50, 100, and 200 ng of μ300 fragment included during binding. Lanes 6, 7, 8 are 50, 100, and 200 ng of μ400 fragments included during binding. Lanes 9, 10, 11 are 50, 100, and 200 ng of a κ enhancer fragment (κE) included during binding. The κ enhancer fragment was excised from a plasmid that contained the AluI–AluI segment of the J_κ–C_κ intron described by Picard and Schaffner (1984) cloned into the SmaI site of pUC 13 and was a kind gift of N. E. Speck.

competition experiments with subfragments from within μ300 showed that this complex could not be competed away by either μ50 (Figure 2D, lanes 5 and 6), $(\mu 60)_2$ (lanes 7 and 8), or μ70 (lanes 11 and 12), but only by itself (lanes 9 and 10). Thus the dissection of μ300 revealed two distinct and specific regions of binding, one in μ50 (apparently the O sequence) and one in μ70.

Ephrussi et al. (1985) and Church et al. (1985) have used methylation protection experiments to define a set of G residues within the heavy chain enhancer that are specifically resistant to methylation by dimethyl sulfate (DMS) in B cells or B cell nuclei. This result led to the proposal that

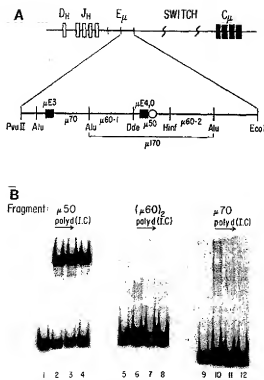


Figure 2. Dissection of the μ300 Fragment to Localize Factor-Binding Sites

(A) Schematic representation. For further dissection, the μ300 segment was cleaved with AluI, DdeI, and HinfI to generate the fragments named μ50, μ70, and μ60 (there are two fragments of approximately 60 bp obtained by restricting the large AluI–AluI piece [μ170] with DdeI and HinfI). The black boxes represent regions of the μ enhancer found to be resistant to methylation by DMS in vivo. Four homologous domains have been identified earlier (Ephrussi et al., 1985), which we refer to as μE1–μE4. Of these, only two, μE3 and μE4, are present in the fragments being analyzed and are located in μ70 and μ50, respectively. The open circle (O) refers to the octamer sequence (ATTTGCAT), which has been found in the promoters of all sequenced V_H and V_L genes and is also found in the μ enhancer. The central G residue in this sequence was also found to be resistant to methylation in whole cells (Ephrussi et al., 1985).

(B) Binding analysis of subfragments of μ300. In each set of four lanes, the first shows the free fragment and the next three shows the effect of carrying out a binding analysis in the presence of increasing amounts (1.2, 2.4, and 3.6 μg, respectively) of the nonspecific competitor poly d(I·C) and a constant amount (8 μg) of protein derived from the EW cell line. $(\mu 60)_2$ represents a mixture of the two 60 bp fragments. Labeled fragments are as follows: lanes 1–4, μ50; lanes 5–8, $(\mu 60)_2$; lanes 9–12, μ70.

(C) Competition experiments to show that the complex formation on μ50 is sequence-specific. Binding reactions were carried out in a final 15 μl volume with 8 μg of a nuclear extract derived from the human myeloma cell line 8226 with 0.1–0.3 ng of end-labeled probe (~12,000 cpm) and 2.5 μg poly d(I·C). Lane 1, free fragment; lane 2, no competitor DNA added; lanes 3–7, binding reactions in the presence of 50 ng of different competitor fragments (as noted above the lanes) added prior to addition of the nuclear extract. κpr is a 300 bp StaNI–StaNI fragment derived from the promoter region of the MOPC 41 κ gene. It extends from position −330 to position −30 relative to the cap site (Queen and Baltimore, 1983). The conserved octanucleotide sequence (ATTTGCAT) is located between base pairs −59 and −66. SV40E is a 170 bp fragment containing both 72 bp repeats of SV40 from the HpaII site on the late side. κE is a fragment containing the κ enhancer (see Figure 1B for details).

(D) Competition experiments showing that competitor formation on μ70 is sequence-specific. Lane 1, free fragment (0.2–0.3 ng, 10,000 cpm); lane 2, binding reaction in a final volume of 15 μl in the presence of 1.5 μg of poly d(I·C) and 12 μg of nuclear extract derived from the cell line EW; lanes 3–12, binding reactions as described for lane 2, but also containing unlabeled competitor DNA derived from the μ enhancer in the amounts shown above each lane.

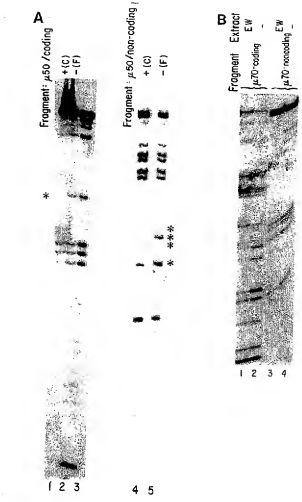


Figure 3 Methylation Interference Experiments to Define the Binding Sites of the Proteins That Interact with μ50 and μ70

The asterisks indicate the location of G residues whose methylation by DMS specifically inhibits the binding of a factor to its cognate sequence.

(A) The μ50 fragment was end-labeled at the DdeI site (0.2–0.3 ng/10,000 cpm) on the coding strand. A typical preparative reaction was done with 80,000–100,000 cpm in an EW nuclear extract. Lane 1, nucleoprotein complex band (C) analyzed after elution from a preparative, low ionic strength polyacrylamide gel and piperidine treatment. Lane 2, free fragment band (F) from the same binding reaction. Lane 3, G ladder generated from end-labeled μ50, not exposed to any proteins. Analysis of the noncoding strand after labeling at the HinfI site. Lane 4, nucleoprotein complex band (C). Lane 5, free fragment band (F) from the same binding reaction.

(B) The AhdI–AluI μ70 fragment was subcloned into the SmaI site of pUC 13 (pμ70). The plasmid was restricted with BamHI and end-labeled for coding strand analysis or restricted with EcoRI and end-labeled for noncoding strand analysis. Binding reactions were carried out in EW nuclear extracts. Lanes 1 and 2, analysis of the G residues on the coding strands of the complex band (lane 1) and the free fragment band (lane 2) obtained after a preparative binding and low ionic strength gel electrophoresis. Lanes 3 and 4, analysis of the G residues on the noncoding strand in the nucleoprotein complex band (lane 3) and the free fragment band (lane 4) following preparative binding and low ionic strength gel electrophoresis.

(C) Summary of the methylation interference (in vitro) and the methylation protection (in vivo) experiments used to define protein binding sites within the μ enhancer: the relevant regions of the μ50 and μ70 fragments are shown with the coding strand on top in the orientation in which they appear in the μ enhancer. The circles above the letters show the G residues that were found to be protected against methylation by DMS in vivo (Ephrussi et al., 1985). The encircled Gs are the ones whose methylation interferes with protein–DNA interaction in vitro (dotted circle indicates partial interference).

tissue-specific DNA binding proteins were responsible for this decreased accessibility of the reagent to DNA. The protections fell into four clusters, the DNA sequences of which were sufficiently homologous to derive a consensus sequence for the binding of a putative factor (Church et al., 1985). All four binding sites (μE1–μE4) fall within the 700 bp XbaI–EcoRI fragment; however, μ300 retains only two complete binding domains (μE3 and μE4) along with the O sequence. The μ70 fragment contains the complete μE3 domain and the factor binding in vitro could be the same as that detected in vivo. Therefore it was unexpected that the μ50 fragment containing μE4 (and O) (Figure 2A) should not compete for binding to μ70 (Figure 2D, lanes 5 and 6). In case this was due to the competitor fragment predominantly binding NF-A at the octamer site and thus making it unavailable as a competitor for μ70, we carried out binding reactions and competitions using a partially purified binding protein, generated by chromatography of the crude extract on heparin-Sepharose, that contained μ70 binding activity and was significantly depleted of NF-A. Even with this fraction, μ50 and μ170 failed to compete successfully for the interaction between μ70 and its binding protein (data not shown), thus strongly implying that the binding sites defined in vivo as μE3 and μE4 are not equivalent. Furthermore, when μ50 or μ170 were end-labeled and incubated with column fractions active in μE3 binding, no specific nucleoprotein complexes were seen. Our current level of analysis does not allow us to distinguish whether μE4 has a much lower affinity for the μE3 factor or whether it binds a different factor. Similarly, the μE1 domain (isolated as a HinfI–PstI fragment) does not compete for the factor binding to μ70. However, in this case, the μE1 fragment itself, when end-labeled and analyzed, does generate a discrete nucleoprotein complex that is not effectively competed away by μ70 (Weinberger et al., 1986). The lack of cross-competition implies that μE1 and μE3 are also not equivalent and, furthermore, do not interact with the same nuclear factor. We will refer to the factor binding to the fragment μ70 as NF-μE3 (an abbreviation for nuclear factor interacting with the μE3 sequence).

To determine the location of the binding sites within individual fragments we have used the technique of methylation interference. End-labeled DNA fragments were par-

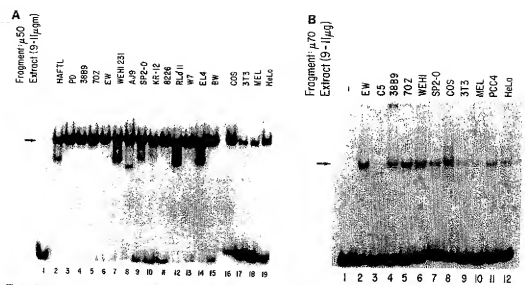


Figure 4. Analysis of μ50 and μ70 Binding in Extracts Generated from a Variety of Lymphoid and Nonlymphoid Cell Lines

(A) End-labeled μ50 was incubated with 9–11 μg of protein from various cell extracts and 2.5 μg of poly d(IC) in a 15 μl binding reaction, followed by electrophoretic analysis. The major complex that has been characterized by competitions (Figure 2C) and methylation interference (Figure 3A) is indicated by the arrow. Lane 1, free fragment; lanes 2–19, binding analysis in various extracts. The cell lines used to derive the extracts are indicated above each lane. Briefly, HAFTL is a very early pre-B mouse cell line; PD, 3B9B, and 70Z are mouse pre-B cell lines; EW is a human B cell line; WEHI 231 and AJ9 are mouse B cell lines; SP2-0 is a mouse myeloma; KR12 and 8226 are human myelomas; RL 11, W7, EL4, and BW are various T cell lines; COS is a monkey cell line; 3T3 is a mouse fibroblast line; MEL is a mouse erythroleukemia line; HeLa is a human cervical carcinoma cell line.

(B) Binding of a μ70 in various extracts under the same conditions as in (A). The characterized complex is indicated by an arrow. Lane 1, free fragment; lanes 2–12, binding in various extracts.

tially methylated on guanines using DMS. Methylated DNA was then used for a binding reaction with crude extracts, and the complex was resolved from the free fragments by electrophoresis. Piperidine cleavage (Maxam and Gilbert, 1977) of eluted fragments was followed by electrophoresis through 12% polyacrylamide–urea sequencing gels. If any of the methyl groups introduced by reaction with DMS interfered with the binding of a specific protein then that molecule of DNA will be selectively missing in the complex form and subsequently in the corresponding G ladder. The method therefore allows the identification of G residues making intimate contacts with the protein. When the μ50 DNA fragment was used in such an experiment, the free fragment generated a characteristic G ladder (Figure 3A, lanes 2 and 3) and the complex form was specifically depleted of DNA molecules carrying a methyl group at the G residue indicated by the asterisk (lane 1), which lies in the middle of the O sequence. This further implies that the NF-A protein is involved in the binding because the interaction appears to be specifically mediated by its cognate sequence. Presumably, modification of this key G residue seriously impedes the formation of a stable complex between the protein and its cognate sequence. Methylation of a second G residue (Figure 3A, lane 1, lowest of the triplet) also appeared to partially inhibit complex formation. Both of these residues have been shown to be protected against methylation by DMS in vivo (Ephrussi et al., 1985). On the noncoding strand, methylation of the G residue in the middle of the octamer sequence only partially inhibited complex formation (Figure 3A, lane 4, asterisk) whereas modification of any of the three A residues (marked by asterisks) abolished binding completely (lane 4). Interestingly, however, none of the other G residues protected in vivo in this region of the μ enhancer appears to be involved in complex formation as measured by this methylation interference assay (Figure 3C). Therefore, if these protections in vivo are due to the binding of a protein, this factor is different from NF-A and is not binding to the μ50 fragment in vitro.

On the μ70 fragment several G residues were identified as being important in forming intimate contacts with the binding protein (Figure 3B). On the coding strand, bands due to three Gs (Figure 3B, asterisks) were significantly reduced in intensity in the complex as compared with the free DNA fragment (compare lanes 1 and 2); on the noncoding strand two Gs were significantly affected (compare lanes 3 and 4). In Figure 3C, open circles above the sequence indicate the residues identified by Ephrussi et al. (1985) to be protected against methylation in vivo whereas the encircled Gs are the ones identified by us in vitro. The pattern of protection and interference on the μ70 fragment over the consensus sequence is strikingly similar in vivo and in vitro, indicating that the protein identified here may be the one that interacts with this sequence in vivo. Analogous to μ50, however, a second set of protections seen in this region in vivo was not observed in vitro. Interestingly, several G residues in the complex (Figure 3B, lane 1) appear to be more intense than the corresponding residues in free DNA (lane 2). This may mean that some of the modifications allow better interaction between the DNA

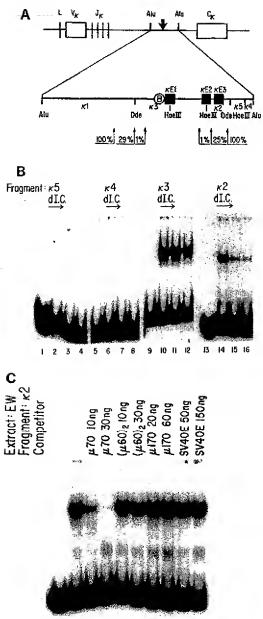


Figure 5. Dissection and Binding Analysis of the κ Enhancer

(A) Schematic representation of the essential 475 bp AluI–AluI fragment containing the κ enhancer as defined by Picard and Schaffner (1984). The vertical arrow represents the approximate location of a DNAase I hypersensitive site in the $J_κ$–$C_κ$ intron. This enhancer was further dissected by cutting with DdeI and HaeIII to generate the fragments κ1 through κ5, which were then used as probes in binding assays. The black boxes represent sequences homologous to the consensus sequence derived by Church et al. (1985) based on the protections against methylation by DMS observed in vivo on the μ enhancer. The lowest line summarizes the results of Queen and Stafford (1984), who have carried out fine deletion mapping of the κ enhancer. Thus deletions from the 5′ end to about 20 bp past the DdeI site cause a significant loss of enhancer function. Similarly 3′ deletions extending 10–15 bp beyond the second HaeIII site cause a significant loss of enhancer function. B locates the B cell–specific protein binding site described in this paper.

(B) Binding analysis of subfragments of the κ enhancer. In each set of four lanes, the first shows the free fragment followed by binding analyses in the presence of increasing amount (1.2, 2.4, and 3.6 μg) of the nonspecific competitor poly d(IC) and a constant amount (~8 μg) of protein derived from an EW nuclear extract. Labeled fragments are as follows: lanes 1–4, κ5; lanes 5–8, κ4; lanes 9–12, κ3; lanes 13–16, κ2.

(C) Competition analysis to show that κ2 interacts with the same nuclear factor that binds to μ70. Lane 1, free fragment; lane 2, binding of end-labeled κ2 (0.2–0.3 ng, 10,000 cpm) in the absence of competitor DNA. Typical reaction contained 10 μg protein from EW nuclear extracts and 2 μg of poly d(IC) in a 15 μl volume. Lanes 3–10, binding reactions essentially as described above, but additionally containing unlabeled competitor DNA fragments derived from the μ enhancer (refer to Figure 2A) or the SV40 enhancer (Figure 2C) in the amounts shown above each lane.

(D) κ3 binds a specific factor in EW nuclear extracts. Lane 1, free fragment; lane 2, binding of end-labeled κ3 (0.1–0.3 ng, 10,000 cpm) in the absence of competitor DNA. A typical reaction contained 10 μg protein from EW nuclear extracts and 2.5 μg of poly-d(IC) in a 15 μl volume. Lanes 3–12, binding reactions in the presence of competitor DNAs added in the amounts shown above each lane. Refer to Figure 2C for derivations of the κpr, κE and SV40E fragments.

and the specific protein presumably by subtly altering the DNA conformation.

Tissue Specificity of the Factors Detected

To ask whether the proteins we have identified are limited to expression only in B cells, we have screened extracts from a large number of cells (Figure 4). Complexes that comigrated with the ones generated and characterized in the B cell line EW were observed on both the fragments (μ50 [Figure 4A] and μ70 [Figure 4B]) in all the cell lines examined. (Comparison of independent extracts indicates that estimates of the abundance of proteins in different cell lines using this assay are not meaningful.) Although the complex generated in each extract has not been further characterized, we interpret this data as indicating that both of these factors are not tissue-specific. A second

complex (having a greater mobility) was observed with the μ50 fragment that appears to be restricted to B and T cells only and will be described fully later (Staudt et al., unpublished results).

Dissection of the κ Enhancer

An enhancer element has also been identified in the major intron of the κ light chain gene. Picard and Schaffner (1984) showed that the enhancement activity can be localized to a 500 bp AluI–AluI fragment, and Queen and Stafford (1984) have shown that deletion of the 5′ AluI–DdeI fragment has a minimal effect on enhancer activity, restricting the enhancer to 275 bp from DdeI to the 3′ AluI site (Figure 5A; the black boxes represent sequences identified by Church et al. [1985] as homologous to the series of E domains detected in the μ enhancer). Fragments were generated by cutting with DdeI and HaeIII (see Figure 5A) and assayed for binding in the presence of increasing amounts of poly d(IC) as a nonspecific competitor. Fragments κ4 and κ5 appeared negative (Figure 5B, lanes 1–8), while κ3 and κ2 formed complexes (lanes 10–12 and 14–16). κ1 is too large a fragment to be reliably assayed and has not been further dissected yet. Preliminary results show that the internal undesignated HaeIII fragment does not contain any specific binding sites either. The competition pattern for κ2 was strikingly similar to what had been observed earlier with the μ70 fragment: relatively large amounts of μ400, the Moloney leukemia virus enhancer, the SV40 enhancer, or the κ promoter (containing the O sequence) did not compete for binding, while μ300 and the κ enhancer did (data not shown). Because κ2 contains a putative E box identified by sequence comparison (as does μ70), we competed away its binding with smaller fragments from μ300 (Figure 5C). The complex is specifically competed away by the addition of unlabeled μ70 during the incubation (Figure 5C, compare lanes 3 and 4 with lane 2), but not by the addition of $(\mu 50)_2$ (lanes 5 and 6), μ170 (lanes 7 and 8), or the SV40 enhancer (lanes 9 and 10). Furthermore, the protein that binds to this sequence cofractionates with NF-μE3, the μ70 binding activity, through two sequential chromatographic steps (heparin agarose and DEAE–Sepharose) (data not shown). We conclude that the same sequence-specific protein (NF-μE3) binds to both fragment μ70 and fragment κ2; therefore, at least one common protein interacts with both the μ and the κ enhancers.

The κ3 complex (Figure 5D, arrowhead) failed to be competed away by μ300 (lanes 3 and 4), μ400 (lanes 5 and 6), or a κ promoter-containing fragment (lanes 7 and 8). The complex, however, was specifically competed away by both the complete κ enhancer (Figure 5D, lanes 9 and 10) and the SV40 enhancer (lanes 11 and 12). The band below the major κ3 complex was seen at variable intensities in different experiments and failed to compete even with the complete κ enhancer in this experiment and has not been further investigated. The observation that the SV40 enhancer specifically competes for binding of this factor suggests that an identical stretch of 11 nucleotides (GGGGACTTTCC) shared with the SV40 enhancer may be responsible for the binding.

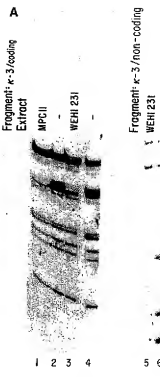
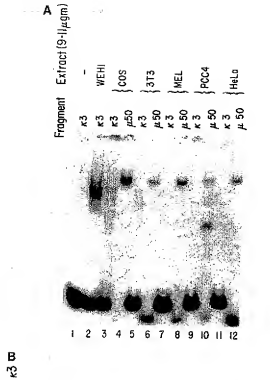


Figure 6. Methylation Interference Analysis of the Nucleoprotein Complex Generated on κ3

(A) Preparative binding reactions were carried out with partially methylated κ3 end-labeled at the DdeI site in two different extracts: MPC11 (mouse myeloma) and WEHI 231 (mouse B cell line). The complex and free fragment bands were eluted from a low ionic strength polyacrylamide gel, treated with piperidine, and analyzed by electrophoresis through a 12% sequencing gel. Lanes 1 and 3, G ladder corresponding to the nucleoprotein complex bands generated in MPC11 extracts and WEHI 231 extracts, respectively. Lanes 2 and 4, G ladder corresponding to the free fragment isolated after binding in MPC11 and WEHI 231 extracts, respectively. Lanes 5 and 6, analysis of the G residues on the noncoding strand in the nucleoprotein complex band (lane 5) and the free fragment (lane 6) following preparative binding and low ionic strength gel electrophoresis. G residues whose methylation interferes with nucleoprotein complex formation are indicated by the asterisks.

(B) Summary of the methylation interference (in vitro) experiments used to define the G site within the κ enhancer. The relevant region of the κ3 fragment is shown with the coding strand on top. The encircled Gs are the ones whose methylation interferes with protein DNA interaction.

We have localized the binding site of this factor on the κ3 fragment by carrying out methylation interference experiments. In two different extracts, methylation at three of a stretch of four G residues on the coding strand within the sequence shared with SV40 completely abolished binding (Figure 6A, compare the complexed fragments in lanes 1 and 3 with the free fragments in lanes 2 and 4; asterisks indicate the position of Gs whose methylation seriously interferes with binding). On the noncoding strand, methyla-



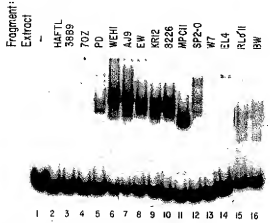


Figure 7. Analysis of κ3 Binding in a Variety of Lymphoid and Nonlymphoid Cell Extracts

(A) κ3 binding reactions carried out in five nonlymphoid lines. Lane 1, free fragment; lane 2, binding in a cell line positive for this factor (WEHI 231). A typical reaction had 0.1–0.3 ng (10,000 cpm) of labeled fragment together with 2.5 μg of poly d(I·C) and 9–11 μg of nuclear extract. Odd numbered lanes 3–11, binding reactions in the same conditions as above in a variety of extracts generated from nontymphoid lines as noted above each lane (refer to Experimental Procedures for a description of these lines). Even numbered lanes 4–12, binding of μ50 (which detects a ubiquitous factor) under the same conditions, serving as a positive control for each extract.

(B) κ3 binding reactions in lymphoid cell extracts. Lane 1, free fragment. Binding reactions were performed, as detailed above, in extracts derived from pre-B cell lines, (HAFTL, 38B9, 70Z, and PD) (lanes 2–5); B cell lines (WEHI 231, AJ9, and EW) (lanes 6–8); plasma cell lines (KR12, 8226, SP2-0, and MPC11) (lanes 9–12); and T cell lines (lanes 13–16). Note that the mobility of the complex formed differs slightly between extracts derived from mouse cells (e.g., AJ9, WEHI 231) or human cells (EW, KR12, and 8226). The significantly altered mobility in the MPC11 extracts is probably due to proteolysis, since many other binding sites also show higher mobility complexes in this extract.

tion of three G residues (Figure 6A, lane 5, asterisks) inhibited complex formation. Thus the binding site was (shown in Figure 6B; circled G residues are those determined by methylation interference experiments to be important for complex formation) localized toward one end of the κ3 fragment (Figure 5A, represented by B). This result also served to explain the specific competition observed earlier with the SV40 enhancer. Interestingly, deletion mapping of the κ enhancer was shown that sequences within the κ3 fragment are extremely important for enhancer function (Queen and Stafford, 1984).

The tissue range of this factor was examined by carrying out binding analysis with κ3 using extracts from a variety of cell lines. Nucleoprotein complex formation with κ3 was detected in a mouse B cell line (Figure 7A, lane 2) but not in 5 other non–B cell lines (odd numbered lanes from 3–11). Even numbered lanes in Figure 7A show that the ubiquitous factor detected by μ50 is present in all of these cell lines and serves as a positive control for the experiment. The factor therefore appears to be restricted in expression to B lymphoid cells. We then examined extracts made from cells at various stages of B cell differentiation (Figure 7B). Interestingly, κ3 binding protein was detected in the pre–B cell line PD (Figure 7B, lane 5), which spontaneously rearranges its κ genes (Lewis et al., 1982); two mouse B cell lines (WEHI 231 and AJ9, lanes 6 and 7); one human B cell line (EW, lane 8); two mouse myeloma cell lines (MPC11, SP2-0, lanes 11 and 12); and two human myelomas (KR12 and 8226, lanes 9 and 10). However, it was not apparent in a very early pre–B cell line (HAFTL, Figure 7B, lane 2) and two standard mouse pre–B cell lines (38B9 and 70Z, lanes 3 and 4). Thus this factor appears to be not only tissue-specific and limited to cells of the B lymphoid lineage, but also stage-specific within that lineage. For these reasons, we refer to the binding site for this factor as the B site and refer to the factor as NF-κB.

In the series of extracts examined, the presence of the NF-κB factor is strikingly correlated with κ gene expression, but there is one apparent discrepancy concerning its stage specificity. Cell line PD, which was derived by Abelson murine leukemia virus transformation of adult bone marrow cells (Rosenberg and Baltimore, 1976) and undergoes κ light chain rearrangement in culture, had the factor. Cell line 70Z, which is apparently further along the B cell differentiation pathway relative to PD, having already completed rearranging its κ light chain genes (Maki et al., 1980), had no detectable factor. We believe that these may not be contradictory because 70Z cells do not actively transcribe the κ locus and do not have the DNAase I hypersensitive site (Parslow and Granner, 1982) in the $J_κ$–$C_κ$ intron that has been correlated with κ gene expression. For PD, however, the κ enhancer is active after transfection (Speck and Baltimore, unpublished results); and the DNAase I hypersensitive site associated with the κ enhancer can be detected (Sen and Baltimore, unpublished results).

Table 1. Summary of Immunoglobulin Enhancer–Binding Factors

Factor	Binding Site(s)	Tissue Distribution
NF-A	Octamer sequence (ATTTGCAT) in V(H), V(L) promoters and μ enhancer	Ubiquitous (B cell specific component)
NF-μE3	E3 site in μ enhancer	Ubiquitous
NF-μE1	E1 site in μ enhancer	Ubiquitous
NF-κB	B site in κ enhancer	κ-Producing B cells only

E1, E2, E3, etc., refer to the E homology identified by Ephrussi et al. (1985). NF-μE1 has been identified by Weinberger et al. (1986).

Discussion

We have detected interaction of multiple factors (summarized in Table 1) with Ig μ and κ enhancer sequences using an electrophoretic mobility shift assay. Within the 300 bp PstI–EcoRI fragment of the μ enhancer, two sites have been localized. One is an octamer (O) sequence (ATTTGCAT) that is also conserved upstream of all heavy and κ chain variable region genes and appears to bind the ubiquitous NF-A factor. The second sequence coincides with one of the motifs (μE3) shown by Ephrussi et al. (1985) and by Church et al. (1985) to be bound to a factor in B cells. Both of the factors were detected in a broad range of cells and therefore did not appear tissue-specific. There appears also to be a tissue-specific factor that can bind to the O sequence (Staudt et al., unpublished results). The μE1 and μE4 sequences that have close homology to the μE3 sequence competed poorly, if at all, for binding to the μE3 sequence, implying that the sequence specificity of the μE3-binding protein is quite exquisite. A different protein has been shown to bind to μE1 (Weinberger et al., 1986).

Our dissection of the κ enhancer has revealed two binding sites. One of these, κE3, corresponds closely in sequence to one of the μ sites, μE3 (see Figure 8), and its binding protein appears to be NF-μE3, the factor that binds to μE3. Thus there is at least one protein that interacts with both the μ enhancer and the κ enhancer. The second site, B, is the most unique one we have found because NF-κB, its binding protein, is restricted in appearance to cells that ordinarily express κ chains. It is not present in the μ enhancer, but is found in the SV40 enhancer.

Although all of the data presented here involved crude nuclear extracts as the source of protein, preliminary results of chromatographic fractionation are consistent with the proposal that at least three separable factors interact with the immunoglobulin enhancers.

The enhancers are defined partly by their ability to enhance transcription when present in either orientation relative to a promoter. It might therefore be thought that their binding sites should have dyad symmetry. The E series of sequences (those suggested by Ephrussi et al. [1985] to be homologous) do contain an element of dyad symmetry.

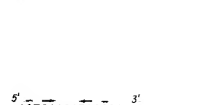


Figure 8. Comparison of E Domains from the μ Enhancer and the κ Enhancer

μE1–μE4 were defined by Ephrussi and Church in the μ enhancer on the basis of methylation protection experiments in vivo (Ephrussi et al., 1985). κE1–κE3 were identified within the κ enhancer as being homologous to the consensus sequence derived by comparing μE1–μE4 (Church et al., 1985) (see Figure 5A). The arrows over the sequence point out a mini dyad axis of symmetry within each domain. (Those also include in every case the most conserved residues in the consensus sequence [Church et al., 1985].) In the fragments used by us to dissect the enhancers, μE3 is completely present within the μ70 fragment, μE4 within the μ50 and μ170 fragments, and κE3 within the κ2 fragment.

As illustrated in Figure 8, there is a motif of CA/TG in all of these sequences with a separation of two or three bases. One of the next outer two bases also has a symmetric counterpart giving a dyad of three out of four bases in each sequence. The two sequences that appear to bind the same factor (μE3 and κE2) also have identical internal two base spacers of TG. Of those that do not compete for binding with μE3 and κE2, μE1 has a three base spacer and μE4 has a spacer of GG. Another related sequence (κE2) within the κ enhancer also has a GG dinucleotide within its inverted repeat but has yet to be analyzed. We have been unable to detect any binding to μE4 thus far, but μE1 appears to have a specific binding factor (Weinberger et al., 1986). The inability of such closely homologous sequences (particularly μE4) to compete for binding to μE3 suggests that these nuclear binding proteins may have restriction enzyme–like specificity in their binding. Perhaps some of the enhancer-binding proteins belong to families of related proteins with slightly different binding specificities.

Two of the identified binding sites have no homology either to the E series, or to each other. One, the O sequence from the μ enhancer (ATTTGCAT), is bound by NF-A, a non-tissue-specific factor, but also is bound by a factor found only in cells of the B lymphoid series (Staudt et al., unpublished results). The other, the B sequence from the κ enhancer (found around the sequence GGGGACTTTCC), binds to NF-κB, a factor specific to cells that express κ chains. Ignoring the nonspecific factor that binds to the O sequence, it might appear that each enhancer has E-related elements that bind to nonspecific factors and unique elements that bind to specific factors and that could be responsible for the tissue specificity of the individual enhancers. As discussed elsewhere (Singh et al., 1986), the existence of a nonspecific factor that binds to the O sequence might relate to the use of this site for

regulating transcription by promoters such as those for the U1 and U2 RNAs.

There is an apparent discrepancy between the in vivo binding data (Mercola et al., 1985; Ephrussi et al., 1985) and the in vitro data we have accumulated. Ephrussi et al. (1985) and Church et al. (1985) find that the E sites and the O site are protected against methylation in B cells but not in fibroblasts. We find that NF-μE3 and NF-A are present in fibroblastic and other nonlymphoid cells. It would therefore appear that the mere presence of a factor is not sufficient for it to bind in such a way as to generate protection of the G residues at the site in vivo; in all likelihood, the protein is not bound to a site except in specific cells. This implies that for binding to occur, a given segment of DNA may have to be "activated," a process that may involve making chromatin accessible to the binding factors. It is possible that tissue-specific binding proteins play the role of activation and therefore open the DNA to interaction with nonspecific transcription enhancing proteins. In vitro, where naked DNA is used for assay, such interactions would not be evident and the factors would all appear to be equivalent DNA binding proteins.

Although one might expect that at least some of the sequences to which factors bind in the tissue-specific Ig enhancers would be unique to these structures, most of the sequences have close relatives in other regulatory elements. The two sequences most likely to be involved in tissue specificity—the O sequence and the B sequence—are both found in the SV40 enhancer, and that viral sequence will compete for binding of factors to O and B in vitro. Transcriptional potentiation by the SV40 enhancer can be competed away by the μ enhancer both in vivo and in vitro, suggesting that a common factor may interact with both of these sequences (Mercola et al., 1985; Sassone-Corsi et al., 1985). A possible explanation for this could be that when the SV40 enhancer is activated in a non-B cell, it uses some other sequences or binding sites for its activity, whereas when it is activated in a B cell, it uses, for instance, its homolog of the NF-κB binding site. In this model, the SV40 enhancer could be a mosaic of different sequence motifs recognizing a number of different factors, only a subset of which is needed in any one cell to generate function.

In previous studies using the mobility shift assay, we have determined the site of binding using a variant of the DNAase I footprinting method (Singh et al., 1986). In the present study we have used a methylation interference assay both because it allows a higher resolution analysis of the binding site and because we found that many complexes cannot be assayed by the footprint method. For those complexes that do not yield a footprint, there appears to be too rapid an equilibration between complexed and free DNA to allow complexes to be treated with DNAase and then resolved by electrophoresis. For instance, the half-life of the nucleoprotein complex with the μ70 fragment is less than a minute (Sen, unpublished observation). In the methylation interference protocol, DNA that is methylated will not rebind eluted proteins, and therefore the bound complexes never contain DNA methylated at a residue critical to the binding reaction.

Experimental Procedures

Extracts and Cell Lines

Nuclear extracts were made from the following tissue culture cell lines exactly according to the protocol of Dignam et al. (1983) and usually contained 6–12 mg/ml of protein: HAFTL, Harvey sarcoma virus transformant (Pierce and Aaronson, 1982), which presumably represents an early stage in B cell differentiation because it is still in the process of carrying out D_H–J_H rearrangements at the immunoglobulin heavy chain locus (Desiderio and Baltimore, unpublished results); 38B9 and PD, Abelson murine leukemia virus transformants, which are pre-B-like because they either contain a rearranged (VDJ) heavy chain locus (PD, Lewis et al., 1982) or are in the process of assembling their heavy chain genes (38B9, Yancopoulos et al., 1984); 70Z, mouse pre-B cell line; WEHI 231 and AJ9, mouse B cell lines containing functionally rearranged heavy and light chain genes; EW 36, human EBV-negative Burkitt lymphoma; KR12 and 8226, human myelomas (gift from Dr. C. M. Croce); SP2-0 and MPC 11, mouse myelomas; EW5147, W7, EL4, and RLon1t, mouse T cell lines; COS, monkey cell line; 3T3, mouse fibroblast cell line; MEL, mouse erythroleukemia cell line; P C4, mouse embryonic carcinoma line; HeLa, human cervical carcinoma cell line.

Plasmids were constructed as follows. The 300 bp PvuII–EcoRI fragment of the μ enhancer was digested with AluI, and the fragments were subcloned into pUC13 cut with SmaI to yield pμ70 (containing the 70 bp AluI–AluI insert) and pμ170 (containing the 170 bp AluI–AluI insert). See Figure 2A for a restriction map of the relevant region.

Competitor DNA corresponding to the various μE boxes were prepared as follows: pE1, by BamHI–PvuII digestion of pμEc (a plasmid containing the 220 bp HinfI–HinfI fragment of the μ enhancer, subcloned into SmaI-cut pgC13, which was a gift from Dr. J. Weinberger); pE3, by EcoRI and BamHI digestion of pμ70; pE4, by HinfI–DdeI digestion of the insert obtained by cleaving pμ170 with EcoRI and BamHI.

The 475 bp AluI–AluI fragment containing the κ enhancer (κE) was subcloned into pUC13 cut with SmaI. Competitor DNA was prepared by cutting at flanking sites within the polylinker, DdeI and HaeIII were used to generate the various smaller fragments as shown in Figure 5A. The κ promoter (κp) was obtained from a plasmid that contained approximately 300 bp (spanning positions −35 to −330 relative to the cap site of the MOPC 41 κ gene) of an SfaNI fragment that was subcloned into SmaI-cut pSP64 (a gift of Dr. N. E. Speck). Large competitor fragments (greater than 150 bp) were isolated from low melting point agarose gels by soaking the minced gel slice in elution buffer containing 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. After a 6–8 hr incubation at 37°C, the supernatant was extracted once with phenol and once with chloroform and the DNA was precipitated by adding 2.5–3 volumes of ethanol. Competitor DNA was quantified by comparison to standard weights of DNA either after electrophoresis through agarose or after spotting onto an agarose plate. Radioactive probe was obtained by end-labeling dephosphorylated DNA with [γ-^{32}P]ATP in the presence of polynucleotide kinase (Boehringer Mannheim Biochemicals). Typical specific activities ranged from 30,000–70,000 cpm/ng of DNA.

Gel Binding Analysis

Binding reactions were carried out in 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 4% glycerol for 20–30 min at room temperature. Poly d(IC) was added as a nonspecific carrier, and a typical reaction contained 10,000 cpm (0.2–0.5 ng) of end-labeled DNA with 9–11 μg of extract (which were added last). Following binding, the mixture was electrophoresed through a native 4% polyacrylamide gel (acrylamide; bisacrylamide ratio 30:1) containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA. The gel was preelectrophoresed for 2 hr at 11 V/cm. Electrophoresis was carried out at the same voltage for 2 hr at room temperature with buffer recirculation. The gel was then dried and autoradiographed with a screen at −70°C. For competition experiments the conditions were exactly as above, except that specific and nonspecific competitor DNAs were included in the mixture (in amounts as detailed in the figure legends) prior to addition of the protein.

Methylation Interference Experiments

End-labeled DNA fragments were partially methylated at the guanine residues, as detailed by Maxam and Gilbert (1977) with the following modification. The reaction was quenched with 1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, and 100 μg/ml of poly d(C). Methylated DNA was precipitated twice, rinsed with 70% ethanol, dried, and taken up in TE (10 mM Tris [pH 8.0] and 1 mM EDTA). For a typical preparative binding reaction, the usual conditions were scaled up 5 to 10 fold. Binding and gel electrophoresis were as above. After electrophoresis the gel was wrapped with Saran wrap and exposed wet for 4–6 hr at room temperature. The complex and free fragment bands were then excised and electroeluted for 1–2 hr (tRNA was added to prevent adsorption of labeled DNA to the membrane) to recover the DNA. Prior to ethanol precipitation, the solution was extracted sequentially with phenol and chloroform. The pellet was rinsed thoroughly with cold 70% ethanol, dried, then redissolved in 100 μl of 1 M piperidine. Base cleavage reactions were carried out for 45 min at 90°C followed by removal of the piperidine by lyophilization. After two additional rounds of lyophilization from water, the products were analyzed by separation by electrophoresis through a 12% polyacrylamide gel in the presence of 8 M urea followed by autoradiography at −70°C with a screen.

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the thymus of TL^b mice. But it is clear that most of these cells are not eliminated even though they interact with products of the TL^b haplotype in the thymus. As a result of this interaction, KN6 TCR-positive cells increase in size, down-modulate their TCR and probably express IL-2 receptor β-chains, which would confer on them the ability to respond to IL-2 alone[14]. This effect of the TL^b product does not reflect an essential positive selection step, because KN6 TCR-positive cells do survive in, and are exported from, the thymus of TL^d transgenic mice. Whereas KN6 TCR-positive cells from TL^d thymi respond to TL^b stimulator cells with IL-2 production and proliferation, KN6 TCR-positive cells from TL^b thymi lose the capacity to produce IL-2 and, as a result, respond to TL^b stimulator cells only in the presence of exogenous IL-2. Therefore the response of KN6 TCR-positive cells from TL^b mice to TL^b stimulator cells is dependent on helper cells that supply IL-2. The lack of such helper cells in TL^b mice could explain the lack of destructive autoimmune responses in TL^b transgenic mice and the lower number of transgenic TCR-positive cells in the periphery of TL^b mice versus TL^d mice. The unresponsive state of the TG-positive cells that is generated in the thymus of the transgenic mice used in the study described here is reminiscent of the state of clonal energy that can be induced in peripheral αβ T cells[15–18], as well as in the thymocytes of $P \rightarrow F_1$ chimaeras[19].

Our study shows that certain T cells may become dependent on helper cells if they encounter self-antigen in the thymus as well as in the periphery. Like self antigen-specific B cells, self antigen-specific T cells are not necessarily harmful to the host. In fact they could have beneficial functions provided that helper cells are absent, which would facilitate their proliferation and differentiation to potentially harmful effector cells in response to host components. □

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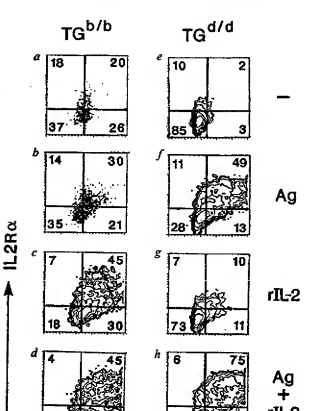

FIG. 3 Blastogenesis and IL-2 receptor α-chain (IL-2Rα) expression of cultured DN thymocytes from KN6 TG mice. DN thymocytes from transgenic mice of TL^b ($TG^{b/b}$) or TL^d ($TG^{d/d}$) haplotypes were cultured in culture medium alone (*a*, *e*), with irradiated TL^b peritoneal cells (*b*, *f*), with rIL-2 alone (*c*, *g*), or with rIL-2 and TL^b stimulator cells (*d*, *h*). After 2 days of culture, cells were stained with 8D6 (anti-V$_\beta$4/8) and PC61 (anti-IL-2Rα) monoclonal antibodies. Shown are the dot-plot histograms (IL-2Rα fluorescence intensity (log_{10} scale) on the vertical axis, and forward scatter (FSC; linear scale) on the horizontal axis of gated 8D6$^+$ cells, and the percentage of cells in each quadrant.

METHODS. Cultured cells were incubated with PC61 monoclonal antibody (ATCC No. T1B222) followed by FITC-coupled purified goat anti-rat IgG antiserum (CALTAG, San Francisco). After a third incubation in 10% normal rat serum, cells were stained with biotin-coupled 8D6 monoclonal antibody followed by streptavidin-phycoerythrin. Flow cytometry was performed according to procedures described in Fig. 1 legend.

A second B cell-specific enhancer 3′ of the immunoglobulin heavy-chain locus

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THE expression of immunoglobulin heavy-chain (IgH) genes is generally thought to be regulated by the combination of the *VH* promoter with the enhancer element which is located in the *JH–CH* intron[1–4]. This is probably an oversimplification: there are cell lines that transcribe IgH genes despite the deletion of the intron-enhancer[5–8]. These findings could imply that other enhancer element(s) exist in the IgH locus[5–11]. Here we show that a strong B-cell-specific enhancer is indeed located at the 3′-end of the rat IgH locus, 25 kilobases downstream of *Cα*. This enhancer should be retained downstream of all rearranged IgH genes, regardless of the *VH* or *CH* segment used. Taken together with analogous findings for the mouse κ locus[12], the results prompt a re-evaluation

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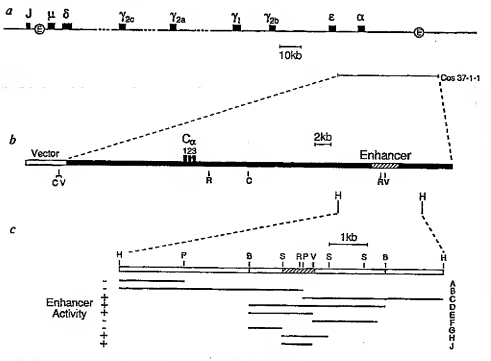

FIG. 1 *a*, The rat IgH locus. Ⓔ denotes the intron and 3′ enhancers. The interrupted line linking *Cγ2c* and *Cγ2a* indicates that the genes have not been physically linked; the gene order derives from analysis of gene deletions in hybridomas[4-6]; the rest of the rat IgH genes have been linked on overlapping cosmids[13]. *b*, Map of cosmid 37-1-1. *c*, Map of the *Hin*dIII enhancer fragment and the enhancer activity of subfragments. Assays were performed as described in the legend to Fig. 2. Restriction enzyme cleavage sites: B, *Bgl*II; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Stu*I; Sm, *Sma*I and V, *Eco*RV. The single *Pst*I site indicated in the shaded enhancer fragment is a doublet.

of the mechanism of regulation of immunoglobulin gene transcription. Furthermore, unlike the intron-enhancer, the IgH 3′ enhancer would become linked to a *c-myc* that rearranges into an IgH switch region. The IgH 3′ enhancer could therefore play a part in the activation of the translocated *c-myc* genes in rat immunocytomas, mouse plasmacytomas and Burkitt lymphomas.

In screening for an additional enhancer in the IgH locus, we focused our attention on sequences 3′ of $C\alpha$, because this region is likely to be maintained in all rearranged IgH genes regardless of the particular *VH* or *CH* gene used. We used cosmids that covered >30 kilobases (kb) of the 3′ end of the rat IgH locus. The locus of rat (Fig. 1*a*) has striking homology to that of the mouse both in overall organization and in sequence. For example, the rat intron-enhancer and many of the individual *CH* genes show >90% homology to their mouse counterparts[13,14].

To test for enhancer activity, we exploited the fact that the human β-globin gene in plasmid pβ800 (Fig. 2*a*) is only a weak transcription unit in transfected cells unless it is provided with an exogenous enhancer element. We scanned cosmid 37-1-1 for enhancer elements because this cosmid spans $C\alpha$ and the region 3′ of it (Fig. 1*a*). Random *Hin*dIII fragments of Cos 37-1-1 were inserted downstream of the β-globin gene in plasmid pβ800. The DNA from the resulting constructs was transfected into the

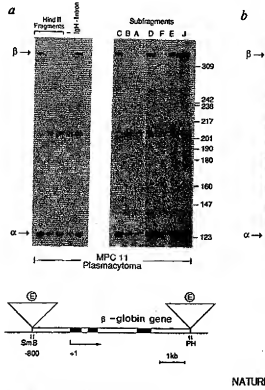

FIG. 2 *a*, Location of the rat IgH 3′ enhancer. Ribonuclease protection assay measurements of human β- and α-globin mRNAs in transfected mouse MPC11 plasmacytoma cells. Cells were transfected with derivatives of plasmids pβ800 (see diagram) or pβ128, along with a reference plasmid that included the human α_2-globin gene. Bands corresponding to correctly initiated β- and α-globin transcripts are indicated. Plasmid pβ800 (gift from K. Weston) consists of the human β-globin gene with 800 nucleotides (nt) of 5′ flanking sequence cloned between the *Hin*cII and *Pst*I sites of plasmid pUC12; plasmid pβ128 includes 128 nt of 5′ flanking sequence. The derivatives of plasmid pβ800 include *Hin*dIII (left panel) or *Pst*I fragments (fragments A, B and C) cloned downstream of β-globin gene, whereas the other fragments were cloned upstream. The mouse IgH-intron enhancer (IgH-Intron; 1-kb *Xba*I fragment) served as a positive control. *b*, Cell-type specificity of the IgH 3′ enhancer (IgH-3′; *Stu*I–*Eco*RV fragment). Parallel assays were performed in HeLa cells and in mouse thymoma cell lines EL4 and BW5147. The SV40 enhancer served as a positive control. Transfection of MPC11 and HeLa cells by calcium phosphate co-precipitation, transfection of T-cell lymphomas by use of DEAE-dextran, and measurements of RNA levels, were carried out as previously described[4,12].

mouse plasmacytoma cell line MPC11 by calcium phosphate co-precipitation along with a human $\alpha 2$-globin plasmid that served as an internal reference. The amount of β- and α-globin messenger RNAs produced by the transfected cells was then measured in ribonuclease protection assays. One of the *Hin*dIII fragments caused a considerable stimulation of β-globin transcription and gave rise to mRNA levels comparable to those achieved with the IgH intron-enhancer (Fig. 2*a*). Mapping of this *Hin*dIII fragment revealed that it contained sites for *Eco*RI and *Eco*RV, which allowed us to identify its position relative to the $C\alpha$ gene (Fig. 1*b*). Further fragmentation of the cosmid and of clones derived from it demonstrated that enhancer activity was in a 0.7-kb *Stu*I–*Eco*RV fragment located 25 kb downstream of the $C\alpha$ exons (Fig. 1*c*). This IgH 3′ enhancer has the classic properties of a transcriptional enhancer element in that it is active in both orientations and functions both upstream and downstream of the test gene. (The *Hin*dIII fragments and *Pst*I fragments A, B and C were assayed downstream of the β-globin gene, whereas fragments D–J were assayed upstream. Similarly, fragments H and J were assayed in opposite orientations to each other). To test whether the 3′ enhancer possessed ubiquitous or lymphoid-specific activity, we introduced the plasmid constructs into fibroblasts and T-cell lymphomas. Although activity was easily detected in plasmacytoma, no activity was detected in either HeLa cells or in the mouse T-cell lymphomas BW5147 and EL4 (Fig. 2*b*). Therefore, like the IgH-intron enhancer[4], the 3′ enhancer is active in plasmacytoma, but not active in T cells or nonlymphoid cells in these transient transfection assays.

The sequence of the *Stu*I–*Eco*RV fragment which encompasses enhancer activity was determined and is shown in Fig. 3. It includes a stretch of GA repeats preceded by oligo(G-T).

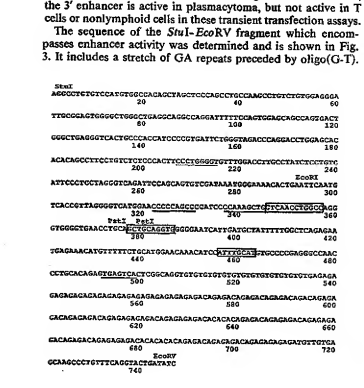

FIG. 3 Sequence of the rat IgH 3′ enhancer (IgH-3′). The sequence of both strands was determined by the dideoxy-chain-termination method. Homologies or identities to regions of the mouse IgH-intron enhancer (IgH-intron) are boxed; those to AP-1, AP-2 or AP-4-binding sites[25] are underlined.

Comparison of the IgH 3′ enhancer with the intron of the gene for IgH and simian virus 40 (SV40) enhancers revealed homologies that span several notable motifs. In particular, a perfect copy of the octanucleotide (ATTTGCAT) is present at position 456. The octanucleotide element is found in the IgH-intron enhancer, is an essential component of V_H promoters[4] and is sufficient in appropriate assays to confer lymphoid-specific gene expression[15–17]. There is also a good match to another region of the mouse intron-enhancer that is implicated in lymphoid-specific transcriptional activation, that is the region surrounding the μE2 and μE5 motifs[18,19], as well as significant homology to a region surrounding the NF-μE1 binding site. As regards SV40 comparisons, there is a match to the consensus binding site for transcription factor AP-1, and homologies to the AP-2- and AP-4-binding sites. Analysis of cosmid subfragments A, B and C (Fig. 1*c*) indicates that the most important motifs are likely to lie across, or 3′ of, the *Pst*I site at position 378; this would include the octanucleotide, μE5 and AP-1 motifs. But functional assays are clearly necessary to evaluate the significance of the homologies.

The rat IgH-intron enhancer shows B cell-specific enhancer activity and >90% sequence homology to its mouse counterpart (ref. 13, and our unpublished observations). Therefore, the rat IgH locus, like the mouse κ light-chain locus[12], contains both a J–C intron-enhancer and a 3′ enhancer. We do not believe that the presence of several enhancers is peculiar to the rat IgH locus; preliminary studies indicate that a 3′ IgH enhancer is also present in the mouse IgH locus. The presence of several enhancers in the immunoglobulin gene loci could simply reflect redundancy. From transgenic mouse studies[20], however, it seems that the combination of V_H promoter with IgH intron-enhancer is not sufficient to give properly regulated IgH expression *in vivo*. In fact, in the context of the accessibility model of immunoglobulin gene rearrangement[21], it is possible that the intron-enhancer is primarily concerned with the regulation of V_H–D–J_H joining, and that the 3′ enhancer is implicated in transcriptional regulation at later stages of differentiation.

The existence of an enhancer at the 3′ end of the IgH locus could explain the transcriptional activation of the translocated *c-myc* genes characteristic of several lymphoid malignancies. In

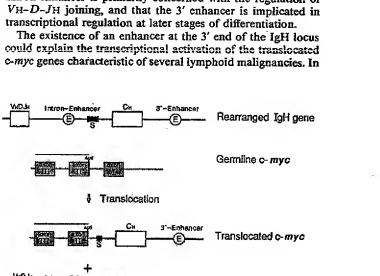

FIG. 4 The structure of a typical *c-myc*/IgH locus translocation. The precise structure of the translocation depends on the tumour, with the favoured incoming C_H gene being $C\varepsilon$ in rat immunocytoma, $C\alpha$ or $C\gamma$ in mouse plasmacytoma, and $C\mu$ or $C\gamma$ in Burkitt lymphoma. Similarly, the *c-myc* breakpoint can either be upstream of *c-myc* or it can be within *c-myc*, separating the noncoding exon (exon 1) from the coding region (the AUG initiator codon is in exon 2). In most cases, the breakpoint in the IgH locus is within a switch (S) region. The translocation therefore separates the IgH-intron enhancer from the coding region of *c-myc*, because they end up on reciprocal products of the translocation. But the translocated *c-myc* will become linked to an IgH 3′ enhancer, which could therefore play a part in *c-myc* transcriptional activation.

many human Burkitt lymphomas, mouse plasmacytomas and rat immunocytomas, *c-myc* is translocated into the IgH locus[22,23]. But the location of the breakpoints on the IgH locus-bearing chromosome indicates that the IgH-intron-enhancer is unlikely to be involved in the activation of the translocated *c-myc* allele, because the protein-coding region of *c-myc* and the IgH enhancer end up on reciprocal products of the translocation[24]. As shown in Fig. 4, however, the translocated *c-myc* allele would typically be linked to the IgH 3′ enhancer, and this enhancer could therefore be implicated in the origin or maintenance of B-cell neoplasias. □

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A single origin of phenylketonuria in Yemenite Jews

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PHENYLKETONURIA (PKU) is a metabolic disease caused by recessive mutations of the gene encoding the hepatic enzyme phenylalanine hydroxylase (PAH). The incidence of PKU varies widely across different geographic areas, and is highest (about 1 in 5,000 live births) in Ireland and western Scotland, and among Yemenite Jews. A limited number of point mutations account for most of the PKU cases in the European population. Here we report that a single molecular defect—a deletion spanning the third exon of the PAH gene—is responsible for all the PKU cases among the Yemenite Jews. Examination of a random sample of Yemenite Jews using a molecular probe that detects the carriers of this deletion indicated a high frequency of the defective gene in this community. Although the deleted PAH gene was traced to 25 different locations throughout Yemen, family histories and official documents of the Yemenite Jewish community showed that the common ancestor of all the carriers of this genetic defect lived in San'a, the capital of Yemen, before the eighteenth century.

Without PAH activity in the liver, the hydroxylation of the amino acid phenylalanine to tyrosine cannot occur, and phenylalanine accumulates in the blood and is degraded at an excessive

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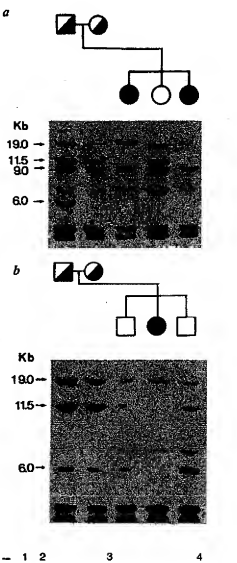

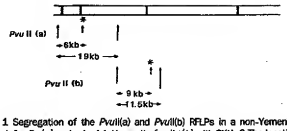

FIG. 1 Segregation of the *Pvu*II(a) and *Pvu*II(b) RFLPs in a non-Yemenite Jewish family (*a*) and a Jewish Yemenite family (*b*) with PKU. *C*, The location of the two RFLPs relative to exons 1–4 of the *PAH* gene. Normal segregation of the *Pvu*II(a) alleles (6.0 and 19.0 kb) is observed in both families, whereas none of the *Pvu*II(b) alleles (9.0 and 11.5 kb) are present in the DNA of the Yemenite patient. Southern blotting, hybridization and autoradiography were performed as previously described[26]. The cDNA probe phPAH247 (ref. 5) was labelled using the random priming method[25]. Asterisks denote polymorphic restriction sites. Symbols: square, male; circle, female; filled symbol, PKU patient; half-filled symbol, PKU carrier.

Enhancer Complexes Located Downstream of Both Human Immunoglobulin Cα Genes

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Summary

To investigate regulation of human immunoglobulin heavy chain expression, we have cloned DNA downstream from the two human Cα genes, corresponding to the position in the mouse IgH cluster of a locus control region (LCR) that includes an enhancer which regulates isotype switching. Within 25 kb downstream of both the human immunoglobulin Cα1 and Cα2 genes we identified several segments of DNA which display B lymphoid-specific DNase I hypersensitivity as well as enhancer activity in transient transfections. The corresponding sequences downstream from each of the two human Cα genes are nearly identical to each other. These enhancers are also homologous to three regions which lie in similar positions downstream from the murine Cα gene and form the murine LCR. The strongest enhancers in both mouse and human have been designated HS12. Within a 135-bp core homology region, the human HS12 enhancers are ~90% identical to the murine homolog and include several motifs previously demonstrated to be important for function of the murine enhancer; additional segments of high sequence conservation suggest the possibility of previously unrecognized functional motifs. On the other hand, certain functional elements in the murine enhancer, including a B cell–specific activator protein site, do not appear to be conserved in human HS12. The human homologs of the murine enhancers designated HS3 and HS4 show lower overall sequence conservation, but for at least two of the functional motifs in the murine HS4 (a κB site and an octamer motif) the human HS4 homologs are exactly conserved. An additional hypersensitivity site between human HS3 and HS12 in each human locus displays no enhancer activity on its own, but includes a region of high sequence conservation with mouse, suggesting the possibility of another novel functional element.

The regulation of human immunoglobulin heavy chain gene expression is incompletely understood, despite clinically significant conditions in which specific isotypes are inappropriately up- or downregulated, e.g., allergies due to inappropriate IgE response, and various forms of immunodeficiency associated with low IgA expression. Clearly, cytokines and interactions between B and T cells play a role in regulating isotype switching, and *cis* elements in the IgH gene locus which mediate these effects have been documented in the murine and human promoters of the sterile transcripts associated with each heavy chain constant region gene (1, 2). However, in the mouse an additional control region which contributes to regulation of isotype switching has been reported to lie downstream from Cα, and the corresponding region of the human heavy chain locus has not yet been investigated.

The existence of a regulatory region downstream from murine Cα was originally inferred when it was found that plasmacytomas which had undergone spontaneous deletions of the only heavy chain enhancer then known, which lies in the intron between JH and Cμ, nevertheless remained capable of high level immunoglobulin secretion (3–6). Conversely, a myeloma subclone which retained the intronic enhancer but lost a segment of DNA downstream from the murine Cα gene was found to have markedly reduced its heavy chain gene expression (7). A systematic search in the homologous region of the rat heavy chain locus revealed an enhancer (8), and a homologous mouse enhancer designated 3′αE was found soon after (9, 10) positioned ~16 kb downstream from Cα. The mouse and rat 3′αE segments lie in opposite orientations and are flanked by inverted repeats (9). In addition to the 3′αE, Matthias and Baltimore also reported a weak enhancer in mouse lying only 4 kb downstream from Cα (Fig. 1 and reference 11).

F.C. Mills and N. Harindranath both made substantial contributions to this work.

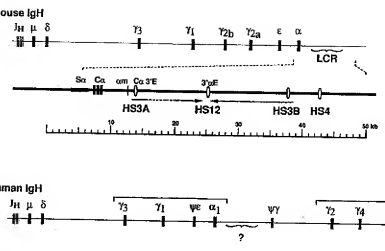

Figure 1. Comparison of IgH locus of mouse and human. Line *A* shows a map of the murine IgH locus, from which the region downstream from Cα is expanded in line *B*. The murine enhancers designated Cα3'E (11) and 3'αE (9) are shown as vertical ovals, along with the DNase I hypersensitivity site designations (12). We have distinguished the two copies of HS3 sequence as HS3A and HS3B; these are included in a large palindrome (arrow) that flanks HS12, according to the sequence analysis of Chauveau and Cogné (13). Line *C* shows the human IgH locus, illustrating the γ-γ-ε-α duplication units (*brackets*) and the possibility of two regions homologous to the murine LCR.

More recently, Madisen and Groudine (12) analyzed B cell–specific DNase I hypersensitivity downstream from Cα and identified four hypersensitive sites. HS1 and HS2 fall in the previously described 3'αE, whereas HS3 and HS4 lie further downstream and identify two new regions with somewhat weaker enhancer activity in transient transfection assays. The HS3 sequence is almost identical to that of the enhancer described by Matthias and Baltimore but has an inverted orientation. This reflects the fact that the sequence surrounding the HS12-3'αE is present in the mouse in a long inverted repeat which includes HS3 sequences at both ends (Fig. 1 and reference 13). When constructs containing HS3, HS12, and HS4 linked to a reporter gene were transfected into a B cell line, subsequently isolated stable transfectants were found to express the reporter gene in a position-independent manner. This suggested that the three enhancer sequences (HS12, HS3, and HS4) acted together as a locus control region (LCR)[1]. LCRs, first defined in the globin locus (14), activate large domains of chromatin in vivo (100 kb in the human β globin locus), and, as components of DNA constructs in transgenic mice, support gene expression proportional to the number of integrated copies. In contrast, integrated gene constructs lacking LCR sequences are variably expressed, depending on the positions of integration. LCRs typically contain several DNase I hypersensitive sites, which often represent DNA with enhancer activity. In addition to the LCRs found in the β globin and mouse IgH loci, LCRs have also been described as associated with macrophage-specific lysozyme, CD2, and α/β TCR loci (15).

Analyses of the regulatory regions downstream from murine Cα have identified several motifs which bind specific transcription factors to mediate different aspects of regulation of enhancer function. The 3'αE has been found to activate transcription strongly in plasmacytomas, but only weakly in earlier B lymphoid cells. Part of this developmental change is attributable to a motif known as E5, which matches the "E-box" consensus binding site (CANNTG) characteristic for members of the basic helix-loop-helix family of transcription factors. The contribution of the E5 site to enhancer activity is inhibited in early stages of development by the dominant negative nuclear regulator Id3, which is expressed in early B lineage cells but downregulated in plasma cells (16). At least four other motifs in the 3'αE have been reported to contribute to enhancer activity specifically in plasmacytomas, motifs whose contribution in B cells is inhibited by BSAP (the B cell–specific activator protein), which disappears as B cells mature to plasma cells. These sites include αP (17), the octamer motif (ATGCAAAT; reference 18), a κB-like site (16), and a G-rich sequence (19). In B cells, BSAP prevents the binding of the transcriptional activator NF-αP to the αP site, and causes the octamer, G-rich, and κB-like motifs to exert an active repressive influence on transcription (17, 19–21).

Apart from the motifs mediating upregulation of the 3'αE during maturation to plasma cells, a response element in the enhancer for activation induced by B cell receptor cross-linking has been traced to partially overlapping sites for the ETS family member Elf-1 and for members of the AP-1 transcription factor family (22). Two other motifs in the enhancer have been proposed to contribute to its regulation, but are less well documented: the μE1 and the μB motifs, which were first noted in the rat 3'αE and which are partially conserved in mouse. The HS3 and HS4 enhancer regions of mouse have been studied in less detail, but the HS4 enhancer apparently contains functional Oct-1 and BSAP binding sites (23).

A role for the 3'αE in isotype switching was revealed by experiments in which this region was replaced by a neomycin resistance gene through homologous recombination in embryonic stem cells which were then used to reconstitute the B cell population in RAG-2 knockout mice. The re-

[1]*Abbreviations used in this paper:* BAC, bacterial artificial chromosome; BSAP, B cell–specific activator protein; HSE, heat shock element; HSTF, heat shock transcription factors; LCR, locus control region.

sulting B cells showed normal V(D)J recombination but marked deficiencies in switching to IgG2a, IgG2b, IgG3, and IgE in vitro, whereas expression of IgM and IgG1 was normal (24). This observation suggests that the enhancer exerts isotype-specific effects on switch recombination, possibly through its regulation of germline transcription of the different isotypes before switch recombination.

Because the enhancer regions lying downstream from the mouse Cα gene have been found to be important for heavy chain gene expression and isotype switching, there has been considerable interest in determining how homologous regions might regulate immunoglobulin gene expression in humans. The human heavy chain locus includes two γ-γ-ε-α segments (25, 26), apparently the product of a large duplication in the primate lineage (27). Isotypes from the upstream duplication, comprising the γ3-γ1-ψε-α1 constant region genes, are generally expressed at a much higher level than those of the downstream γ2-γ4-ε-α2 duplication. The existence of two Cα genes in humans suggests the possibility that two 3′ enhancer complexes may regulate the locus, one downstream from each Cα gene; differences in these complexes could contribute to the differential regulation of the two duplications. Moreover, individuals who have a ψε-α1–ψγ deletion on one chromosome show reduced expression of the downstream γ2 and γ4 genes on that chromosome, indicating that this deletion may have removed a region which exerts distal control over at least some of the human heavy chain genes (28). Finally, the possibility that there are two 3′α enhancer complexes makes the human IgH locus an attractive candidate for study because there could be interactions between two adjacent LCRs, a situation which has not been described in any other system.

Several laboratories have attempted to characterize sequences lying downstream from the two human Cα genes, but technical difficulties have impeded this work. Gene walking downstream from the Cα genes has been difficult, apparently because of a segment of 20-bp tandem repeats which lies almost immediately downstream of the most 3′ exon of both Cα genes. These tandem repeats, described independently by three laboratories (29–31), include the sequence GATC recognized by the isoschizomer restriction enzymes Sau3A and MboI. Since commercial human DNA libraries in λ phage have been constructed using genomic DNA fragments generated by partial MboI/Sau3A digestion, the repeated Sau3A sites downstream of the Cα genes make it unlikely that library clones isolated by hybridization to Cα probes will contain DNA downstream from the repeats.

As an initial step toward defining the role of global control regions in the activation of the human IgH genes, our laboratory has sought to determine: (*a*) are enhancer complexes located downstream from the human Cα genes; and (*b*) how do any such human enhancers correspond to the regulatory sequences described downstream from the murine Cα gene? Towards this end we have successfully applied several strategies other than gene walking from Cα to obtain DNA clones extending downstream from the human Cα genes, enabling us, by functional analysis and sequencing, to characterize enhancer regions 3′ of each Cα gene homologous to those of the mouse HS12, HS3, and HS4.

Materials and Methods

Cloning of 3′α Regions from Human Genomic DNA. To obtain DNA between Cα1 and the previously reported ψγ pseudogene which lacks associated Sγ sequences, we initially sought clones containing the pseudogene, which should hybridize to a Cγ probe but not to an Sγ probe. We screened a commercial library of partial MboI-digested human placental DNA in the λFixII phage vector (Stratagene, La Jolla, CA) with a Cγ probe; probe f (Fig. 2), a 7-kb HindIII fragment, was isolated from a pBR322 plasmid clone originally derived from λ phage CH-Ig-H-g-11(32) (Health Science Research Resources Bank, Osaka, Japan; e-mail: hsrrb@nihs.go.jp). Cγ+ plaques were replated and duplicate plaque-lift filters were hybridized with the Cγ probe and an Sγ probe (1,100-bp KpnI to PstI fragment containing human Sγ2; reference 33). Southern blots of BamHI-digested DNA from eight candidate clones (Cγ+, Sγ−) were hybridized with a ^{32}P-labeled oligonucleotide Pseudogam-1 (see Table 1 for sequences of oligonucleotides) specific for the ψγ hinge region at 48°C in hybridization buffer (1 M NaCl, 0.1 M sodium phosphate, pH 7.0, 10% dextran sulfate, 10 mM EDTA, 1% SDS), followed by washing at 48°C in 1× SSC, 10 mM EDTA, 0.1% SDS. This analysis identified three overlapping ψγ+ clones (ψγ-25, ψγ-2, and ψγ-38) extending over an ~30-kb region containing the ψγ gene (Fig. 2).

To obtain clones for the expected homologous region downstream of the Cα2 region, two probes derived from the farthest upstream ψγ clone (ψγ-25) were used to rescreen two duplicate plaque lifts of the λFIXII library. Probe b (Fig. 2) was a 4.5-kb fragment extending from the 5′ end of ψγ-25 to the first internal EcoRI site. Probe e was a 1.3-kb PCR fragment which lies ~2.5 kb upstream from ψγ and which was obtained by amplification from a ψγ-25 subclone containing a 10.5-kb EcoRI-XbaI fragment which extends from the farthest downstream EcoRI site in ψγ-25 to an artificial XbaI site at the 3′ end of the phage insert. Primers used for this amplification were a reverse sequencing primer (Table 1) and elkb2, a primer based on sequence data from a ψγ-25 subclone. This probe contains a sequence homologous to a gene encoding the transcription factor *elk-1*, which is known to lie on the X chromosome (34). Clones EG3-6 and EG3-5 were selected for the following properties: they hybridized to probe e but not to a Cγ probe (as expected for DNA downstream of Cα2), and they had restriction maps distinct from that of the α1 locus and that of the *elk-1* gene on the X chromosome, as established by genomic Southern blots of DNA from a mouse/human somatic hybrid cell line (NIGMS repository No. GM06318B; Coriell Institute for Medical Research, Camden, NJ) carrying the human X chromosome as its only human DNA. Clone H3E-1 was isolated on the basis of hybridization with probe b but not with a Cγ probe. By restriction maps and Southern blots, clones H3E-1, EG3-6, and EG3-5 were found to overlap with each other, forming a contig of ~30 kb.

From genomic Southern blots, the phage contigs downstream of the two Cα genes were found to lie ~20 kb away from clones containing the respective Cα genes. To bridge these gaps, a bacterial artificial chromosome (BAC) library of human genomic DNA (Genome Systems, Inc., St. Louis, MO) was screened using both a Cα membrane exon probe (Fig. 2, probe a) made by amplification from the Cα1 plasmid GMA5 using primer 5′TM-A1

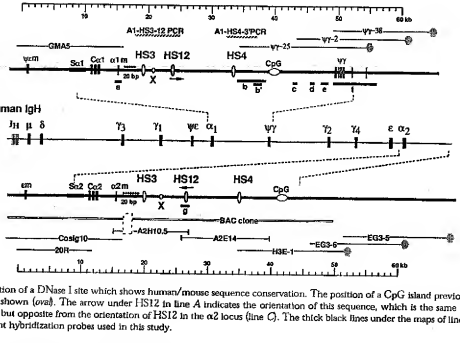

Figure 2. Regulatory loci downstream of human Cα1 and Cα2. Lines *A* and *C*, based on this study, show an expanded map of the region downstream of Cα1 and Cα2, respectively, as well as available DNA clones, which are shown above (α1) or below (α2) the line; phage clones are marked with diagrammatic phage heads, while the subclones of PCR-amplified segments A1-HS3-12 and A1-HS4-3′ are drawn with hatched lines; and a BAC clone is drawn as a double line, containing a deletion (*dashed box*). Vertical ovals mark DNase I sites demonstrating enhancer activity and named according to the homologous murine HS sites. A series of small triangles identifies the 20-bp repeats located downstream from human Cα genes. X marks the position of a DNase I site which shows human/mouse sequence conservation. The position of a CpG island previously identified by Southern blotting is also shown (*oval*). The arrow under HS12 in line *A* indicates the orientation of this sequence, which is the same as that of the homologous mouse HS site, but opposite from the orientation of HS12 in the α2 locus (line *C*). The thick black lines under the maps of lines *A* and *C* (*single lower case letters*) represent hybridization probes used in this study.

in combination with primer 3′TM-B (35) and a probe extending from the most 5′ HindIII site in ψγ-25 to a position ~1,300 bp upstream (Fig. 2, probe b′). A single BAC clone was obtained, spanning ~120 kb, from ~20 kb upstream of Cγ2 to 35 kb downstream from the Cα2 membrane exon. From this clone, designated BAC 11771, two subclones were prepared: a 10.5-kb HindIII fragment (A2H10.5) and an overlapping 14-kb EcoRI fragment (A2E14). Fragments of these subclones were found to have sequence homology to murine HS12, HS3, and HS4, and enhancer activity in a transient transfection assay (see Results).

Because several library screens were unsuccessful in identifying phage or BAC clones covering the gap between Cα1 and our downstream phage contig, selected DNA segments in this region were amplified by PCR. Southern blots of human genomic DNA indicated that the HS12 regions near α1 and α2 lie in opposite orientation. This inversion made it possible to selectively amplify α1-derived sequence from genomic DNA using two primers which both corresponded to the sense strand of the α2 locus. Amplification with the upstream primer SA2.5-A2 and the downstream primer SA2.1-A2 was performed using Taq polymerase XL (PE Applied Biosystems, Foster City, CA) for 32 cycles (94°C for 1 min, 61°C for 2 min, and 72°C for 10 min), and yielded the 5.5-kb A1-HS3-12 PCR fragment (Fig. 2). From this fragment a 982-bp α1 HS-3 product was amplified using primers SA2.5A and SA2.6A; and 964 bp α1HS12T and 892 bp α1HS12B fragments were amplified using primers SA2.1A and SA2.2B. For comparisons between enhancer activities of corresponding α1 and α2 fragments, the homologous 1070-bp A2HS12 PCR product was generated with the same primer pair but using plasmid pEH1.3 as template. In experiments seeking the α1 homolog of HS4, amplifications exploiting the inversion were unsuccessful. As an alternative strategy, we prepared an α1-specific genomic template for PCR: a 23-kb HindIII fragment from the human myeloma HS Sultan (American Type Culture Collection, Rockville, MD) which Southern blots indicated extended from the α1 membrane exon to a HindIII site in our ψγ-25 clone, including DNA homologous to HS4. The corresponding regions from α2 fall in 12-kb and 14-kb HindIII fragments. A 23-kb preparative electrophoresis fraction of HindIII-digested DNA from HS Sultan was used as template for amplification with primers SA8.A and SA11.B based on sequence from the α2 locus. The resulting 4.2-kb fragment (A1-HS4-3′PCR; Fig. 2) was cloned into a λZAP Express phage vector (Stratagene), yielding the clone α1HS4-4.5; the cloned insert showed a restriction map consistent with the α1 locus and distinct from the α2 locus. From the 4.2 A1-HS4-3′PCR fragment a 468-bp segment containing the α1 HS4 region was amplified using primers SA.8A and SA.9B; this segment was used for sequence analysis and enhancer studies. With the same primer pair, the corresponding α2 fragment A2-HS4 was generated using plasmid pA2E14 as template in order to enable meaningful α1 versus α2 comparisons of HS4 enhancer activity.

DNase I Hypersensitive Site Analysis. Nuclei were prepared and digested with DNase I according to a previously described protocol (36). K562 and HS Sultan cells (both obtained from American Type Culture Collection) were grown to densities of 5–8 × 10^5 cells/ml. For each experiment, 3–6 × 10^8 cells were harvested, lysed by addition of NP-40, centrifuged through a 1.7 M sucrose cushion, and resuspended in 5 ml; 450-μl aliquots of suspended nuclei were treated with serially diluted DNase I (Boehringer-Mannheim, Indianapolis, IN) to give final DNase I concentrations of 0–8 μg/ml. Nuclei were digested with DNase I for 3 min at 25°C. For one experiment HS Sultan nuclei were digested with the restriction endonuclease SspI (New England Biolabs, Beverly, MA) for 15 min at 37°C. DNase I (or SspI) digestion was terminated by adding 50 μl 1% SDS, 100 mM EDTA. DNA samples were deproteinized for 5–48 h at 37°C using proteinase K (Boehringer-Mannheim) at a final concentration of 100 μg/ml. DNA was purified by phenol/chloroform extraction and ethanol precipitation, resuspended in 50–100 μl deionized water, and DNA concentrations were measured using a Fluorometer (TKO 100; Hoeffer, San Francisco, CA). 5-μg DNA samples were digested for 5–24 h in 50 μl of appropriate restriction enzyme buffer with BglII, EcoRI, or HindIII (New England Biolabs). To

Table 1. *Sequences of Oligonucleotides Used in This Study*

Oligonucleotide name	Sequence
Pseudogam-1	AGATGCCCACCATGTCAAGT
Reverse sequencing primer	AACAGCTATGACCATG
elkb2	TAAGCTGTCTGAGAGAAAGGTTGGGGGAGG
5′TM-A1	CTGTTCACACGAGTCTGGGCCTGG
3′TM-B	TCCAAGAGGTTCCTCCACACTTCC
SA2.5-A2	ggccggtaccGGATCCCGGTTCCTGATCACTG
SA2.1-A2	ggccggtaccCTTCCTGCCAACCTGGGGGCTG
SA2.5A	ggccacgcgtGGATCCCGGTTCCTGATCACTG
SA2.6A	ggccacgcgtCCACAGTCACTGCCAGATGCTC
SA2.1A	ggccacgcgtCTTCCTGCCAACCTGGGGGCTG
SA2.2B	ggccacgcgtGGCTTTTCCCAGTCCTCCTAC
SA11.B	CAGTGCCCCAACCCAGGACGCCAGCC
SA.8A	ggccacgcgtCGCTCGCTGCCCCACTCAGGAGG
SA.9B	ggccacgcgtCTCCTAGCAGGGTCTCCTCCCTGG
β5PR-A	GAGGAGAAGTCTGCCGTTACTGCCC
β3PR-B	GGCACAATCCAGATGCTCAAGGCCC

All primer sequences are presented 5′ to 3′. For certain primers derived from human genomic DNA sequence, additional nucleotides were added at the 5′ end to provide restriction sites; these nongenomic nucleotides are written in lowercase letters, with the restriction site underlined. All oligonucleotides were prepared in the Facility for Biotechnology Research (Center for Biologics Evaluation and Research, Bethesda, MD).

assess the SspI sensitivity of the β globin locus in HS Sultan nuclei, a 1,511-bp human β globin probe was amplified from total human genomic DNA using primers β5PR-A and β3PR-B. Restriction-digested samples, together with ^{32}P-labeled size markers, were electrophoresed, blotted, and hybridized with the probes indicated in the figure legends. After washing the membranes, radioactive images were obtained with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Enhancer Assays. To analyze DNA fragments for enhancer activity, we used the luciferase reporter plasmid pGL3 (Promega Corp., Madison, WI), modified so that the SV40 promoter between BglII and HindIII sites was replaced by a 76-bp Vκ promoter (37) containing an octamer motif and TATA box. This plasmid, named pGL3-Vκ, served as an enhancerless, promoter-only control. Fragments to be assayed for enhancer activity were blunt-ended with Klenow fragments of DNA polymerase and ligated with MluI linkers, or amplified with primers incorporating an MluI (or KpnI) site; the fragments were then cloned into the MluI (or KpnI) site in the polylinker upstream of the promoter in pGL3-Vκ. Plasmid DNAs for transfection were twice purified on a CsCl gradient.

Approximately 5×10^6 cells in mid-log phase were washed with PBS and electroporated with either 5 μg of promoter-only control construct or equimolar amounts of enhancer test constructs along with 5 μg of CMV-β-galactosidase plasmid (pCMVβ; Clontech, Palo Alto, CA) as an internal control. Each plasmid was electroporated in triplicate in 400 μl PBS using an Electro Cell Manipulator (model 600; BTX Inc., San Diego, CA) at 200 V, 900 μF capacitance, and 13 Ω resistance in a 0.2-cm electroporation cuvette. Immediately after transfection cells were returned to culture medium. 24 h after transfection, the cells were harvested, washed with PBS, and lysed in 50 μl reporter lysis buffer (Promega Corp.) at room temperature for 20 min. After centrifugation at 15,000 rpm for 5 min, 15 μl of supernatant extracts were assayed for β-galactosidase using Galacto-Light Plus Chemiluminescent Reporter Assay kit (Tropix Inc., Bedford, MA) according to the manufacturer's instructions. To assay for luciferase activity, 35 μl of cell extracts was incubated with 100 μl of luciferase substrate [470 μM luciferin in 20 mM tricine, 1.07 mM $(MgCO_3)_4 \times Mg(OH)_2 \times H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.33 mM DTT, 270 μM coenzyme A, and 540 μM ATP]. For both β-galactosidase and luciferase assays light output was detected in a luminometer (Dynatech Instruments, Chantilly, VA).

Sequence Analysis. A PCR-based methodology employing ^{32}S-labeled ddNTPs was used for sequencing reactions (ThermoSequenase kit; Amersham Life Science, Arlington Heights, IL). All samples were amplified for 50 cycles (95°C for 30 s; 60°C for 30 s; 72°C for 60 s). Sequencing reactions were electrophoresed on 6% gels, dried, autoradiographed, and read manually. All reported sequences were read on both A and B strands, except for the sequences of the HS12 59-bp repeats, which, despite several attempts employing various strategies, could be read only on one strand.

Results

Cloning DNA Downstream of Cα1 and Cα2. Because attempts to clone the human 3′ Cα regions using strategies based on gene walking from Cα or cross-species hybridization had failed in other laboratories, we used an alternative strategy based on the fact that a Cγ-like pseudogene (ψγ) has been described downstream of human Cα1 (32, 38). We reasoned that we could clone DNA downstream of Cα1 by walking upstream from ψγ. The similarity in the restriction maps reported downstream of the two Cα genes

based on Southern blotting (26) furthermore suggested that these regions would be highly similar to each other. Therefore, probes derived by walking upstream from ψγ should also hybridize to clones containing DNA from downstream of Cα2.

By screening a phage library for clones that hybridized to Cγ but not Sγ, and then screening those clones with a ψγ-specific oligonucleotide (see Materials and Methods), we obtained three overlapping clones spanning ~30 kb (Fig. 2). To obtain clones covering the corresponding region from the α2 locus, the library was then rescreened with probes b and e (Fig. 2). Clones deriving from downstream of Cα2 were selected on the basis of restriction site differences between the α1 and α2 which had been established by analysis of genomic Southern blots and comparisons with our cloned DNA from the α1 duplication. Three clones deriving from the α2 locus and spanning ~30 kb were obtained (Fig. 2).

To estimate the distance between our two phage contigs and the corresponding Cα genes, we performed genomic Southern blot experiments using a panel of restriction enzymes with known sites in the Cα loci and in our contigs. For example, co-migrating BglII bands of ~35 kb were found to hybridize to both probe a (from the Cα membrane exon αm) and probe b (Fig. 2); these experiments suggested a gap of ~20 kb between each contig and the corresponding membrane exon of Cα (data not shown). For the α2 locus, the gap was bridged by a BAC clone (see Materials and Methods). The corresponding regions from the α1 locus have resisted direct cloning and have been obtained by PCR using primers designed from the sequence of the α2 locus, as described in Materials and Methods.

DNase I Hypersensitive Site Analysis. To map potential regulatory sequences downstream of the Cα genes, we used fragments from the cloned DNA downstream of the Cα2 gene to search for DNase I hypersensitivity sites; in cells where such enhancers or promoters are active, they generally are hypersensitive to endonucleases, apparently because binding of transcription factors disrupts the nuclease protection afforded by nucleosomes. Intact nuclei from the human myeloma HS Sultan were incubated briefly with various concentrations of DNase I; DNA samples purified from these treated nuclei were then analyzed using several Southern blot strategies in order to localize the positions of DNase I cleavage. The analyses were complicated by the fact that all of the probes we used hybridized to both the α1 and α2 loci, but various restriction map differences between the two loci allowed us to position all the hypersensitivity sites with respect to restriction sites mapped from our clones.

Fig. 3 *A* demonstrates analyses of DNA from the promyelocyte K562 line, representative of a cell not expressing immunoglobulin genes, and the myeloma HS Sultan line. The DNase I–treated DNA was digested with BglII, which cuts ~1 kb upstream of the membrane exon of both Cα genes; the blots were hybridized with a probe corresponding to this exon. Since the next downstream BglII site is >20 kb away, this strategy can display hypersensitivity sites over this wide distance from the α membrane exon. The blot demonstrates at least seven hypersensitivity sites, which were subsequently assigned to the α1 or α2 locus by other blotting experiments and were named according to sequence similarity to the homologous murine regions as described below. The sites we have tentatively designated α1X and α2X do not correspond to any reported murine enhancer sequence. None of these sites were visible in the DNA from K562, in which the enhancer region is expected to be inactive.

The BglII blot of HS Sultan DNA fails to resolve the two HS4 sites because they are too far away from the BglII sites. The two HS4 sites were resolved by an alternative Southern blot strategy employing EcoRI digests of the DNA from DNase I–digested nuclei (Fig 3 *B*). To determine which of the two resulting bands represented HS4 from the α1 versus α2 loci, we exploited the observation from our sequence analysis that recognition sites for the restriction enzyme SspI lie in each HS4 site. Since many regulatory regions accessible to DNase I are also accessible to restriction endonucleases, we digested HS Sultan nuclei with SspI and localized the cleavage sites by isolating the DNA, digesting with EcoRI, and hybridizing Southern blots with probe b′ as shown in Fig. 3 *B*. From the map positions of the α1 and α2 SspI sites we assigned the SspI hypersensitivity bands as shown in Fig. 3. A control experiment on the same DNA isolated from SspI-digested HS Sultan nuclei demonstrated that SspI recognition sites in the β globin locus were not cut, indicating that the SspI sites associated with HS4 were indeed hypersensitive in HS Sultan cells (data not shown; see Materials and Methods).

Fig. 3 *C* illustrates an experiment which allowed assignment of several HS sites to the α2 locus rather than α1. DNA samples from DNase I–treated HS Sultan nuclei were digested with HindIII and hybridized with a probe for the HS12 site derived from the α2 locus. Although this probe hybridizes to both the α1 and α2 loci, in this DNA the α1 band is ~23 kb, so large that any fragments from this locus that were generated by HindIII and DNase I and which hybridized to the probe would be larger than the 12-kb band representing the α2 locus. Thus all HS bands <12 kb derive from α2. This blot therefore defines the position of the HS12, X, and HS3 sites in the α2 locus. By implication, the other HS sites in the BglII blot of Fig. 3 *A* must derive from α1. (It should be noted that a common allele in other DNA samples shows an additional polymorphic HindIII site which cuts within the 23 kb corresponding to the HS Sultan band; this allele would have confounded the above strategy, but was absent in HS Sultan.)

Enhancer Function. To analyze enhancer activity in the cloned DNA downstream of the Cα2 locus, fragments containing one or more DNase I hypersensitive sites were subcloned into a luciferase reporter gene driven by a Vκ promoter as described in Materials and Methods. Luciferase activity in each sample was normalized to the β-galactosidase activity of the promoter-only control plasmid, and expressed as fold-increase over luciferase activity of that control plasmid.

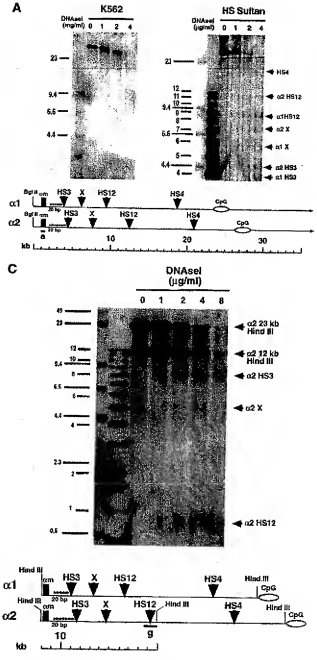

Figure 3. Mapping of DNase I hypersensitive sites in the regions 3′ of the human Cα genes. (*A*) DNase I hypersensitive sites lie downstream from the human Cα genes in the HS Sultan plasmacytoma. DNA samples prepared from DNase I–digested nuclei isolated from K562 promyeloid and HS Sultan myeloma cells were digested with BglII, electrophoresed, blotted, and hybridized with probe a (αm, Fig.1). No DNase I hypersensitive sites are seen in the K562 samples. In contrast, at least seven DNase I hypersensitive sites are observed in samples from HS Sultan plasmacytoma cells. The size of each DNase I–generated band corresponds to its distance from the BglII sites located ~1 kb 5′ of each α membrane exon (*αm*). This mapping strategy does not distinguish between sites in the α1 versus α2 loci; sites are labeled according to their subsequent assignment (see *B* and *C*, and sequence analyses). Due to their large size, bands resulting from DNase I cutting at the α1 and α2 HS4 sites are not resolved in this analysis. (*B*) HS4 sites are accessible to nuclease in both α1 and α2 loci. HS Sultan nuclei were digested with DNase I or SspI restriction enzyme (both the α1 and α2 HS4 sequences contain an SspI site). Purified DNA was digested with EcoRI and hybridized with probe b′, yielding two closely spaced DNase I HS bands, whose sizes correspond to the expected distance between the HS4 enhancers and the downstream EcoRI sites. Furthermore, there are two similarly positioned bands in the samples from SspI-digested nuclei, indicating that both the α1 and α2 HS4 sites are accessible to SspI. (*C*) Assignment of DNase I hypersensitive sites to the 3′ Cα2 region. HS Sultan DNA samples were digested with HindIII and hybridized with probe g (α2 HS12, Fig. 1). Because DNAse I–generated bands from the α1 region which hybridize to this probe are expected to be larger than the 12-kb α2 HindIII fragment, all bands <12 kb must result from DNase I cutting in the 3′ α2 region, with the size of these bands corresponding to their distance from the 3′ end of the 12-kb α2 HindIII fragment. This analysis allows assignment of three DNase I sites to the α2 locus, thus making it possible to assign the other hypersensitive sites seen in the BglII analysis of Fig. 2 *A* to the α1 locus.

The luciferase assays (Fig. 4) revealed strong enhancer activity in the 5-kb SmaI-HindIII fragment (SM5) which was found to contain sequence homologous to murine HS12 (see below). Within this fragment, enhancer activity seemed to be confined to the 1.3-kb EcoRI-HindIII fragment (EH1.3) containing the HS12 site. This segment was further cut into an upstream 0.3-kb EcoRI-PstI fragment (EP300), a 0.3-kb PstI-PstI fragment (P300), and a 0.6-kb PstI-HindIII fragment (PH600). Of these, only the P300 fragment, which contained HS12, showed enhancer activity. However, it should be noted that the enhancer activity of P300 was less than that measured for a larger PCR-generated fragment A2HS12. Thus it is possible that additional elements that do not show intrinsic enhancer activity when

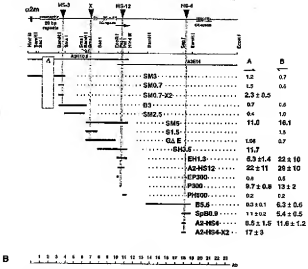

Figure 4. Enhancer activity of selected regions downstream of human Cα1 and Cα2 genes. (*A*) Analysis of the locus downstream of α2, which was studied in detail. The map shows the position of DNase I sites below this are diagrammed the restriction sites defining the boundaries of each fragment tested for enhancer activity by insertion into pGL3-Vκ, transfection into the human myeloma HS Sultan, and assay of resulting luciferase activity, as described in the text. The enhancer activities are given for constructs in the A orientation (the same orientation with respect to transcribed strands of immunoglobulin and luciferase) or the opposite B orientation, where examined. The luciferase activities were normalized to β-galactosidase activity encoded by a cotransfected plasmid, and expressed as fold-increase over the activity of an enhancerless control plasmid. For fragments showing enhancer activity, assays were performed at least in triplicate, and standard deviations are given. (*B*) Comparable analysis of selected fragments amplified from the homologous locus downstream from Cα1.

isolated in constructs can nevertheless augment the activity of the core HS12 enhancer lying in the P300 fragment. Furthermore, the EH1.3 fragment, but not the slightly shorter A2HS12 fragment, showed significantly less enhancer activity in the A orientation, suggesting the possibility of inhibitory sequences located near one of the ends of EH1.3.

Two fragments containing the α2 HS3 site showed no significant enhancer activity in the luciferase construct, but a plasmid in which the HS3-containing 0.7-kb BamHI-SmaI fragment was dimerized showed consistent low activity (Fig. 4, *SM0.7-X2*). The homologous murine enhancer was reported to show greater activity with a c-myc promoter than with an immunoglobulin Vλ promoter (12), but we found no significant increase in enhancer activity when the Vκ promoter was replaced by a human c-myc promoter (data not shown; construct described in Materials and Methods).

Two restriction fragments, B5.5 and SpB0.9, containing the α2 HS4 site demonstrated significant stimulation of luciferase activity, but only when cloned in the B (inverted) orientation (Fig. 4 *A*). When these fragments were oriented so that the DNA strand continuous with the transcribed strand in the luciferase gene corresponded to the transcribed strand of the immunoglobulin gene in genomic DNA (the A orientation), luciferase activity was no greater than that from the promoter-only plasmid. Unexpectedly, a smaller fragment (the 468-bp PCR-generated fragment designated A2-HS4, designed to span the sequence showing the strongest homology to the murine HS4 site) was found to give a strong activation of luciferase activity. The stronger activation of luciferase conferred by the short A2-HS4 PCR fragment compared with that of the longer SpB0.9 fragment (in the same A orientation) suggests that the latter may contain an inhibitory sequence that is either position- or orientation-dependent. This possibility is currently being explored.

Because of the lack of genomic clones spanning the HS3, HS12, and HS4 regions of α1 locus, fragments corresponding to each of these sites were obtained by PCR and analyzed; and, to facilitate α1 versus α2 locus comparisons, the same primer pairs were used to generate corresponding fragments from the α2 locus. As shown in Fig. 4, the activity of each HS site fragment from α1 was similar to that from the α2 locus (compare SM0.7-X2 α2 versus HS3-X2 α1; A2HS12 α2 versus A1HS12T α1; and A2-HS4 α2 versus A1-HS4 α1). The PCR amplification of the HS12 site yielded two fragments, designated A1HS12T (*top*) and A1HS12B (*bottom*), which differ in size by <0.1 kb and may represent alleles (see below). Both fragments showed substantial enhancer activity (Fig. 4 *B*).

Since the mouse 3′α enhancers show different activation patterns during B cell differentiation, with HS4 being activated at the pre–B cell stage, while HS12 and HS3 are only active in mature B cells, we examined the activity of the human 3′ α2 enhancers in a range of human and mouse B cell lines (Table 2). The pattern of activation for the human HS12 enhancer is similar to that of mouse HS12; i.e., human HS12 is inactive in the human pro–B cell line FLEB-14 and the mouse pre–B cell line 18-81, but functions in the human mature B cell line Raji, as well as three plasmacytomas (HS Sultan, human; S194, mouse; and MOPC 315, mouse). HS3 is also inactive in mouse 18-81 pre–B cell line, but shows modest activity in most of the more mature lines tested. HS3 shows surprisingly strong activity in the mouse S194 myeloma, indicating that unknown factors varying between cell lines at similar stages of differentiation can modulate the activity of this enhancer. Finally, the HS4 enhancer shows strong activity in the human pro-B cell line FLEB14, and is also variably active in all of the more mature cell lines (except 18-81) in which this enhancer was assayed in the B orientation.

Sequence Analysis. The nucleotide sequence of all DNase I hypersensitivity sites was determined, revealing ~99% se-

Table 2. *Cell Specifity of Human α2 Enhancer Elements*

Cell line	HS12 (EH1.3)		HS3 (SM0.7)			HS4 (SpB0.9)	
	A	B	A	B	X2	A	B
Human							
FLEB 14 (pro B)	1.9 ± 0.1	1.9 ± 0.1	ND	ND	ND	**2.8 ± 0.2**	**18.4 ± 2.4**
Raji (Mature B)	**3.1 ± 2.2**	**3.2 ± 1.8**	0.7 ± 0.2	1.0 ± 0.3	**2.8 ± 0.5**	1.2 ± 0.1	**2.7 ± 0.5**
HS Sultan (Plasmacytoma)	**5.3 ± 1.4**	**22 ± 10**	1.54	0.59	**2.3 ± 0.5**	1.1 ± 0.2	**5.4 ± 0.5**
Mouse							
18-81 (Pre-B)	0.9 ± 0.2	1.1 ± 0.3	1.2 ± 0.1	0.8 ± 0.2	1.6 ± 0.5	0.5 ± 0.1	0.9 ± 0.2
S 194 (Plasmacytoma)	5.3 ± 0.2	**14.4 ± 1.0**	**8.5 ± 0.8**	**2.6 ± 0.4**	**16.3 ± 0.3**	7.2 ± 1.4	**28.4 ± 2.3**
MOPC 315 (Plasmacytoma)	**5.9 ± 1.1**	**16.9 ± 1.9**	1.4 ± 0.2	0.7 ± 0.2	**2.9 ± 1.9**	1.3 ± 0.6	**5.8 ± 0.9**

The numbers given represent the fold-increase of luciferase activity seen with a promoter-only control plasmid, with standard deviation. Numbers in bold were judged to represent significant enhancer activity.

quence identity between the human α1 and α2 elements, and similarity between the human and mouse enhancers ranging from 74 to 90%.

HS12, the strongest enhancer, showed 90% sequence identity to the homologous murine enhancer over a 135-bp core homology (Fig. 5 *A*). In the α2 locus, four tandem repeats with a 59-bp consensus sequence lie immediately upstream of the HS12 core. However, this sequence has been inverted in Fig. 5 *A*, to facilitate comparison with the homologous α1 sequence in opposite orientation. In the corresponding region of α1 (which, due to the inversion, lies downstream of the core homology region) a 115-bp deletion removes the second and third repeats; the α1HS12B region shows an additional deletion of 70 bp. The core homology region includes several of the functional motifs identified in the murine enhancer: the AP1-Ets site reported to confer responsiveness to B cell receptor cross-linking (22, 39); an exact octamer sequence (ATGCAAAT); and a μE5 site (except in the α1HS12B sequence, in which the μE5 is missing owing to the 70-bp deletion). The sequences of these three motifs from the human HS12 are identical to their murine homologs except for a single base change in the AP1 site which causes the human sequence to exactly match the consensus AP1 site, where the murine motif has one mismatch. The murine element designated μE1 (39), which has never been thoroughly documented even in the murine enhancer, is poorly conserved in the human homologs. Although the murine binding site for NF-κB lies outside the 135-bp region of strongest sequence similarity, a reasonable match to consensus for this element is found in a position roughly homologous to the murine κB site in the α1HS12T and α2HS12 sequences, but is part of the 70 bp deleted in α1HS12B. One of the mouse BSAP sites (BSAP2) is not conserved, but most residues in a second mouse BSAP binding site (BSAP1) are maintained in the human α2 HS12 enhancer. The murine αP site, which binds to an ETS-related transcription factor which augments enhancer activity (17), is not conserved in the human sequences.

The DNase I hypersensitive sites lying <3 kb downstream from the membrane exons of Cα1 and Cα2, roughly in the position of the weak enhancer reported by Matthias and Baltimore (11) and here designated HS3A, were found to contain sequences which are 74% identical to the murine HS3 over a 200 bp core segment. The two human HS3 segments are identical in the 326 bp shown (Fig. 5 *B*), and lie in the same orientation as HS3A. We have assumed that the correct orientation of the murine HS3A sequence is that described by Chauveau and Cogné (13). This orientation is opposite to that of the murine HS3B, which lies downstream from HS12 in the mouse, as described by Madisen and Groudine (12). Of the enhancer/HS sites downstream of murine Cα, HS3 is the least well investigated for functional motifs, in part because of its weak enhancer activity. Independent sequence analysis of the murine HS3A and HS3B regions detected several similarities to octamer motifs, AP1 sites, and consensus E box motifs (CANNTG). The AP1 site identified in Fig. 4 is a precise match to the consensus AP1 binding motif TGANTCA (40) in the two human and two mouse HS3 sequences, and the murine sequence has been shown to bind to c-jun and c-fos in vitro (Neurath, M., personal communication). Similarly, several of the E box consensus motifs in the murine sequence have been shown to bind in vitro to proteins of the HLH family (Neurath, M., personal communication); some of these motifs are conserved in the two human HS3 sequences. The significance of the conserved motifs remains uncertain in the absence of a functional analysis of HS3 sequences.

The DNase I hypersensitivity sites furthest downstream

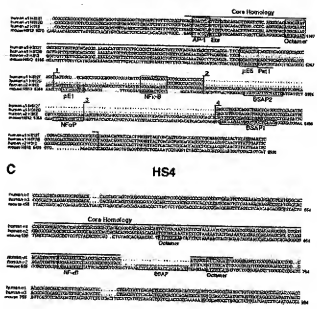

Figure 5. Sequence similarities between human and mouse 3′ α elements. Human-mouse alignments are shown between 3′α enhancers (H12, HS3, and HS4), as well as for the X DNase I site. Nucleotide matches between human α1 and α2 sequences and between one or both human sequences and mouse are indicated by shading. Core homology regions are indicated by a thick line above the sequences. Boxes denote motifs shown to function in mouse as transcription factor binding sites. For HS12, HS3, and HS4, 50–100 bp of sequence flanking the core homology regions are shown. Mouse sequence numbering is 5′ to 3′ with regard to the coding strand of the mouse heavy chain locus. Numbering for mouse HS12, HS3A, HS3B, and X segments is according to reference 13 (EMBL/GenBank/DDBJ accession numbers X96607 and X96608), while numbering for mouse HS4 is according to reference 12 (EMBL/DDBJ/GenBank accession number S74166). (*A*) HS12 sequences (α2 sequence inverted). Overlining highlights the striking 135-bp core segment which is 90% homologous between human and mouse. The sequence alignment has been extended downstream from the core to include additional transcription factor motifs which are functional in mouse. Vertical lines indicate the boundaries of the GC-rich 59-bp repeat units. (*B*) HS3. Comparison of the nearly identical human α1 and α2 HS3 sequences with mouse HS3A and HS3B sequences, which are also nearly identical, shows that there is a 200-bp core segment which is 74% homologous between the mouse and human sequences. (*C*) HS4. Excluding the 25-bp gap containing the mouse HS4 BSAP site, the 145 core HS4 region is 76% homologous between human and mouse. (*D*) X site. Near the center of a 61-bp segment which has 70% human-mouse homology, there is a 20-bp sequence which matches at 19 positions between humans and mice. In both mice and humans this segment contains a consensus HSE (41, 42). The sequences of the human enhancers and X sites are available from EMBL/GenBank/DDBJ under accession numbers AF013718 (α1HS3), AF013719 (α2HS3), AF013720 (α1X), AF013721 (α2X), AF013722 (α1HS12T), AF013723 (α1HS12B), AF013724 (α2HS12), AF013725 (α1HS4), and AF013726 (α2HS4).

from human Cα1 and Cα2 in Fig. 3, which we have designated HS4, are 76% similar to the murine HS4 site over a core 145-bp sequence which spans the three functional motifs demonstrated in murine HS4 (23); see Fig. 5 *C*. The NF-κB motif and the downstream octamer motif in Fig. 5 *C* are both precisely conserved; in the murine HS4 these motifs both contribute to functional enhancer activity (23). In contrast, the BSAP site which upregulates murine HS4 enhancer activity in B cells but downregulates it in pre–B cells is completely absent from the human HS4 sequences.

Lying between HS3 and HS12 in both the α1 and α2 loci are DNase I hypersensitive sites which are not associated with any of the known enhancer elements, but that do map to the position of a 61-bp segment of 70% mouse-human homology. These conserved regions are provisionally designated X sites in part because of their unknown function, and in part because both the human and mouse segments contain XbaI restriction enzyme sites. In mouse this sequence is duplicated as part of the large inverted repeat centered on HS12, so that one copy lies between HS3A and HS12 while a second copy lies between HS12 and HS3B. A segment of $(CA)_n$ repeats is found near the X site in the direction of HS12 at an interval of 80 bp for both mouse X sites (13) and an interval of ~70 bp for the human (data not shown). Within the 61-bp conserved segment, the most highly conserved sequence is a consensus heat shock element (HSE; 41, 42). An HSE could potentially bind heat shock transcription factors (HSTFs), which are known to activate several heat shock response genes (HSP70, HSP90) in response to cellular stress such as heating (43–45). Fragments containing an X site do not appear to dramatically affect enhancer activity in HS Sultan, but may contribute to regulation through mechanisms not captured in our transient transfection assays.

Discussion

Structure of Human 3′ α Regions: Evolutionary Implications. The enhancers clustered 3′ of Cα in the mouse IgH locus can activate the upstream genes, functioning as an LCR. In human IgH locus, arrays of enhancers homologous to those 3′ of mouse Cα are located at two positions within the human IgH locus, 3′ of each Cα gene. On the basis of sequence homology and conservation of restriction

sites between the two human enhancer arrays, it is apparent that these enhancers lie near the 3′ ends of the two duplication units which encompass the γ3-γ1-ψε-α1 and γ2-γ4-ε-α2 gene clusters (25–27), indicating that the 3′α enhancer arrays were present in an approximation of the human arrangement preceding the duplication event that gave rise to the present human IgH locus structure. Moreover, the arrangement downstream of both human Cα genes is 5′-HS3-HS12-HS4-3′, in contrast to the large palindromic structure downstream from mouse Cα that contains a 5′-HS3A-HS12-HS3B-HS4-3′ arrangement (13). Therefore, although an arrangement containing an HS3 enhancer proximal to the Cα membrane exon and an HS4 enhancer farther downstream would seem to have been present in the common ancestor of rodents and primates, the mouse HS3A-HS12-HS3B palindrome probably arose after the primate–rodent divergence. Finally, there is a major structural difference between the 3′α1 and 3′α2 enhancer arrays; namely, that a DNA segment containing HS12 is inverted between the two loci. Using probes containing the 135-bp human HS12 core, it should now be possible to examine DNA from a number of primates for inversion of 3′α2 HS12 relative to 3′α1 HS12; such data may indicate when in evolution the inversion event occurred, and which orientation was present initially in the locus. What caused this inversion? Interestingly, the single mouse HS12 lies in opposite orientation from the rat HS12, and both are flanked by inverted repeats (13) which are known to mediate inversions in other genomic contexts, e.g., in the iduronate-2-sulfatase gene causing Hunter syndrome (46) and in the factor VIII gene (47). Limited Southern blot experiments have not provided evidence for inverted repeats flanking the human HS12 sequences (data not shown). Some hints about the mechanism of the inversion may be found when the inversion breakpoints are identified and sequenced, work currently in progress in our laboratory.

HS12 Structure and Function. The 135-bp HS12 core homology sequence is likely to contain essential motifs important for the strong, late, B cell–specific enhancer activity characteristic of HS12 in mice and humans. Although the function of transcription factor binding sites within the human HS12 core has not yet been demonstrated experimentally, this segment contains sequences nearly identical to the murine AP1, ETS, Oct, and, in α1HS12T and α2HS12, μE5 motifs, all of which are functional in the mouse HS12 enhancer. However, the high degree of sequence conservation in the HS12 core homology extends beyond the transcription factor–binding sites identified in the mouse enhancer, indicating that there may be additional conserved motifs that have not been characterized in either mice or humans.

Despite the fact that a number of other transcription factor motifs whose function has been demonstrated in the mouse HS12 lie outside the HS12 135-bp core homology and are absent in one or more of the human α1HS12T, α1HS12B, and α2HS12 enhancers that we have studied, these enhancers all show roughly equivalent activities. This result suggests that elements missing from these enhancers are not essential for enhancer function in HS Sultan. These inconsistently conserved elements include human sequences corresponding to μE5 and NF-κB sites (absent from α1HS12B) and the BSAP2 site (absent from both α1 alleles). On the other hand, a 1.3-kb α2 EcoRI–HindIII fragment containing the α2 HS12 core plus considerable flanking sequence shows a dependence of enhancer activity on orientation of the fragment (Table 2), suggesting that uncharacterized elements beyond the HS12 core may have some inhibitory function.

Outside the human HS12 core are GC-rich 59-bp repeat units which by themselves do not have enhancer activity in the HS Sultan myeloma (EP300, Fig. 4 *A*), and are not conserved between mice and humans (Fig. 5 *A*). However, it is possible that these repeats contribute to enhancer activity because they are present in the A2HS12 PCR-generated fragment, which gives significantly higher enhancer activity than we observe in the p300 fragment containing the core homology. Deletions of the 59-bp repeats have given rise to apparent allelic polymorphisms, as evidenced by α1HS12T (deletion of the second and third repeats found in α2HS12), α1HS12 (deletion extending from 28 bp 5′ of the first repeat through the third repeat), and other alleles (Harindranath, N., unpublished results).

HS3 and HS4. The other two enhancer components of the mouse 3′α LCR, HS3 and HS4, are weaker enhancers than HS12, but nonetheless are essential for locus control activity (12). Although these elements are less well characterized than the HS12 enhancer, the existing data indicate general human–mouse similarity of the HS3 and HS4 elements, with some notable differences.

In the mouse system, the HS3A element assayed in CAT reporter gene constructs driven by c-fos or thymidine kinase promoters (11) showed weak enhancer activity, although the nearly identical HS3B enhancer showed substantial activity in certain constructs with other promoters tested by another laboratory (12). These disparate results resemble our data on the human α1 and α2 HS3 elements in that single copies and dimers of human HS3 generally gave very low enhancer activity, except in the mouse S194 myeloma in which the same constructs gave substantial enhancer activity comparable to that of HS12 (Table 1). Taken together, these data suggest that HS3, though typically the weakest of the 3′α enhancers, contains uncharacterized motifs that in some cells and/or in combination with certain promoters, can mediate a strong enhancer function.

HS4 is the most downstream 3′α enhancer in both mice and humans, and shows activity intermediate between that of HS3 and HS12. The HS4 enhancer data in the mouse (12, 48), as well as our data on the human α1 and α2 HS4 elements, demonstrate that HS4 is active from the early stages of the B cell lineage onward (Table 2), and thus is qualitatively different from HS3 and HS12. In mouse HS4, there is a binding site for the BSAP, which is expressed in the early B cell lineage. However, in the human α1 and α2 HS4 enhancers, the BSAP site is deleted, indicating that BSAP binding is not an essential feature for HS4 activation

in human pre–B cells. The human HS4 is inactive in the 18-81 mouse pre–B cell line, which was reported to support the activity of mouse HS4 (12). The significance of this difference is not clear; it could be related to the BSAP site deletion in the human HS4, or perhaps to other differences between the mouse and human HS4 sequences.

X Site. The DNase I X sites may represent novel control elements that function together with the HS3, HS12, and HS4 enhancers to activate the IgH locus. Although the significance of the conserved HSE motif is unclear, binding of HSTF protein to an HSE has been shown to be critical for maintaining the DNase I hypersensitivity of the yeast HSC82 gene promoter (49). Heat shock activation of the *Drosophila* HSP70 gene promoter results from binding of HSTF to HSE sites after accessibility of the HSEs has been established by binding of the GAGA protein to adjacent $(GA)_n$ repeats (50). This demonstrated interaction between HSFs and $(GA)_n$ motifs suggests that the location of a $(GA)_n$ repeat region 70–80 bp away from the X site HSE may be of some significance. Furthermore, in the context of IgH gene regulation, it is of interest to note that HSE motifs have been shown to respond to IL-2 and IL-4 (51).

Potential Locus Control Region. In the mouse, it has been demonstrated that when HS3, HS12, and HS4 are linked together in a construct containing the *c-myc* gene and stably transfected into the Raji human B cell line, the c-myc gene is transcribed independent of integration site (12). This observation suggests that the HS1234 combination confers LCR activity, although LCRs have more typically been described based on position-independent transcription of mouse transgenes rather than genes transfected into a cell line. Because the regions 3′ of the human Cα1 and Cα2 genes contain similar HS3, HS12, and HS4 elements that function as enhancers, it is reasonable to hypothesize that these elements also function together in the human system as LCRs. The different arrangement of 3′α enhancers in mice and humans (HS3A-HS12-HS3B-HS4 versus HS3-HS12-HS4) may cause some functional differences in these control regions. Moreover the distance between 3′α enhancers also differs between mice and humans, with the mouse enhancer complex spanning a 30-kb region (13), whereas both the human 3′α1 and 3′α2 enhancers span ~15 kb.

Our finding that arrays of enhancers homologous to those in the mouse 3′α LCR lie downstream of both human Cα genes raises the possibility that differences in the activation of each human γ–γ–ε–α duplication unit result from differences between the putative 3′α1 and 3′α2 LCRs. Even though sequence comparison shows that there is near identity between homologous enhancer elements in the α1 versus α2 locus (Fig. 5), transcription and expression of the upstream heavy chain duplication unit (γ3-γ1-ψε-α1) is greatly elevated relative to the downstream unit (γ2-γ4-ε-α2; reference 2). This difference could result from the fact that the 3′α2 HS12 element is inverted relative to the 3′α1 HS12, and is also at a greater distance from HS3 than in the α1 locus, possibly reducing synergistic interactions between HS3 and HS12. Alternatively, it may be that only the 3′α1 enhancers are activated early in B cell development, possibly falling under the influence of the upstream Eμ enhancer, which itself can function as an LCR (52, 53). In this model, the Eμ and 3′α1 enhancers together would activate a large domain encompassing the first duplication unit, whereas the second duplication unit and the 3′α2 enhancers would fall outside this combined domain. Thus activation of the second duplication unit would depend solely on the 3′α2 enhancers, and expression of genes in this unit might therefore be reduced. Clarification of the basis for this difference will have to await experiments that involve specific deletion of either the 3′α1 or 3′α2 enhancers, as well as studies identifying matrix attachment sites and chromatin insulator elements that define domains within the human IgH region (52–54).

In the work presented here, we have laid the foundation for experimental studies on the activation of the human IgH gene transcription, as well as the regulation of isotype switching. In addition, knowledge of the action of these enhancers on distant constant region genes should contribute to a general understanding of the mechanisms underlying activation of large gene clusters.

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Note added in proof. While this manuscript was under review, related investigations by two other laboratories came to our attention. Chen, C., and B.K. Birshtein (1997. *J. Immunol.* 159:1310–1318.) have described the HS12 enhancers from the α1 and α2 loci; and recently others have characterized the HS3 and HS12 enhancers from the α1 locus (M. Cogné, personal communication).

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Introduction of a μ Immunoglobulin Gene into the Mouse Germ Line: Specific Expression in Lymphoid Cells and Synthesis of Functional Antibody

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Summary

A functionally rearranged μ heavy chain immunoglobulin (Ig) gene was introduced into the germ line of mice. The μ gene encodes a polypeptide which, combined with λ1 light chains, shows a specificity for binding the hapten NP. Four transgenic mice harboring 20–140 copies of the foreign μ gene expressed the gene specifically in spleen, lymph node, and thymus at a high level. Purified surface Ig-positive B cells, Lyt 2-positive mature T cells, and thymocytes transcribed the foreign μ gene at a similarly high level, suggesting that control of Ig gene rearrangement might be the only mechanism that determines the specificity of heavy chain gene expression within the lymphoid cell lineage. No transcription of the foreign μ gene was detected in nonlymphoid tissues with the exception of the heart which expressed the gene at a low level. The transgenic mice had up to 400-fold elevated serum levels of NP binding antibody, which contained a heavy chain with the characteristics of the foreign μ gene. The serum levels of endogenous heavy and light chains in transgenic mice appeared to be the same as in normal mice.

Introduction

Regulation of immunoglobulin (Ig) gene expression is known to occur on different levels. Ig genes are assembled from multiple DNA segments at early stages of B lymphocyte differentiation (reviewed by Tonegawa, 1983). The assembled genes can be transcribed 10^4-fold more actively than their unrearranged counterparts, implying that DNA rearrangement is required for maximal gene transcription (Mather and Perry, 1981). Unrearranged or incompletely rearranged Ig genes, however, although apparently silent in fibroblast cells, are transcribed at a low, but detectable, level in B and even T cells indicating that lymphoid cells contain specific factors that can activate transcription of Ig genes independently of DNA rearrangement (Kemp et al., 1980; Van Ness et al., 1981; Alt et al., 1982). Transcription of the rearranged Ig genes is further regulated during the later stages of the B cell lineage: a dramatic increase in the level of Ig gene transcription occurs when B cells encounter antigen and differentiate into plasma cells in which Ig mRNA accounts for about 5% of the total mRNA (Schibler et al., 1978).

Some of the underlying mechanisms that allow for regulation of Ig gene expression have begun to be elucidated through experiments in which cloned, rearranged Ig genes are transfected into cultured lymphoid or nonlymphoid cells. Such genes are efficiently expressed when introduced into B lymphoid cell lines, but fail to be expressed when transfected into fibroblast cells (Rice and Baltimore, 1982; Gillies et al., 1983; Neuberger, 1983; Queen and Baltimore, 1983; Stafford and Queen, 1983). This implies that the *trans*-acting factors present in the cells of the B cell lineage may interact with *cis*-acting DNA sequence elements closely linked to the transfected Ig genes, to activate their transcription in a tissue-specific fashion. Gene transfer experiments using in vitro modified Ig genes have identified certain *cis*-acting regulatory elements, such as "enhancer" sequences, associated with heavy chain and kappa light chain genes, which appear able to stimulate transcription in B cells but not in fibroblasts (Gillies et al., 1983; Banerji et al., 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984).

To gain a more complete picture of the factors responsible for Ig gene regulation during B cell differentiation a system is required that allows detailed analysis of the developmental regulation and tissue specificity of introduced genes. To this end, we have adopted the approach of gene transfer into the mouse germ line. Another rationale for undertaking such experiments is that they would test models of control of Ig rearrangement (Alt et al., 1980b, 1981; Lewis et al., 1982).

Cloned genes can be transferred into the mouse germ line by microinjection into the pronuclei of mouse zygotes (Gordon et al., 1980; Costantini and Lacy, 1981; Wagner et al., 1981; Brinster et al., 1981). Such microinjected genes frequently integrate into chromosomes, are retained throughout development, and are transmitted to offspring as Mendelian traits. A number of different cloned genes have been transferred into the mouse germ line in this manner. Several microinjected foreign genes have shown a tendancy to be expressed in the correct tissues (Palmiter et al., 1982; McKnight et al., 1983). Recently Brinster et al. (1983) demonstrated that a microinjected rearranged mouse κ light chain gene is efficiently expressed in spleen but not in liver. Thus it appears that in some cases *cis*-acting tissue-specific regulatory mechanisms continue to function after gene transfer into the mouse germ line. We hoped that this approach might also be exploited in the study of the regulation of Ig heavy chain gene expression.

In this paper we describe an analysis of five transgenic mouse lines carrying a cloned, rearranged mouse heavy chain gene. We have examined the transcription of the foreign Ig gene in various tissues of several of these mice, and the data show that the gene is transcribed specifically in B and T lymphoid cells. In addition, we present evidence that four of the five mice synthesize foreign Ig heavy chain

polypeptides which combined with endogenous Ig light chains to form functional antigen-specific antibody. We also find that expression of the microinjected μ gene does not appear to affect the serum level of endogenous Ig heavy chains.

Results

The variable region of the heavy chain gene we chose for introduction into the murine germ line contains a member of the NP gene family of V_H gene segments which is expressed in the BALB/c hybridoma 17.2.25 (White-Scharf and Imanishi-Kari, 1982; Loh et al., 1983). We chose this gene because it encodes a polypeptide with a well-characterized specificity for binding the hapten 4-hydroxy-3-nitrophenyl (NP) (Imanishi-Kari and Maketa, 1974; Karjalainen, 1980). The 17.2.25 heavy chain produces NP binding antibody when it combines with $\lambda 1$ light chains (White-Scharf and Imanishi-Kari, 1982). Because about 5% of the mature B cells in mice contain functional $\lambda 1$ light chains deriving their specificity from a single V_L gene segment (Tonegawa et al., 1978; Selsing et al., 1982), we hoped to monitor the expression of the NP heavy chain gene and the synthesis of a functional polypeptide by measuring the level of NP binding antibody in the serum of transgenic mice. In addition, the immune response against NP in mice is strain-specific (White-Scharf and Imanishi-Kari, 1981, 1982). BALB/c and C57BL/6 mice possess two related, but distinct, sets of dominant NP-specific V_H gene segments, termed V_HNP^a for BALB/c mice and V_HNP^b for C57BL/6 mice. The V_H gene segments of both sets have been cloned, sequenced and their structural differences have been characterized (Bothwell et al., 1981; Loh et al., 1983). For the gene transfer experiment we used C57BL/6 mouse embryos as recipients because we expected that the microinjected V_HNP^a (17.2.25) gene could then be distinguished from the corresponding endogenous NP^b gene copies at the DNA, RNA, and protein levels.

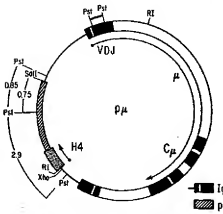


Figure 1. Scheme of the pμ Plasmid DNA

The circular map of the plasmid DNA is shown. The μ gene is depicted as closed (exons), open (introns), and hatched (noncoding sequences) bars; the 5′ and 3′ flanking regions of the μ gene are represented as lines. pBR322 sequences and H4 gene sequences are drawn hatched or dotted, respectively. Restriction sites used for the DNA analysis of the transgenic mice are indicated. The sizes of the Pst I–DNA fragments that hybridize with pBR322 sequences are denoted.

In the 17.2.25 hybridoma, the rearranged V_H gene segment is linked to a $\gamma 1$ constant region. We wanted to introduce a μ gene into the germ line of mice because the μ isotype is activated first in B cell differentiation. For that reason we used a DNA clone, called pLV2, in which the rearranged V_H 17.2.25 region had been fused to the genomic μ gene segment (D. Loh and C. Queen, unpublished data). We isolated the μ gene from this clone and inserted the gene into a pBR322 derived vector (Figure 1). The μ gene included 2 kb of DNA sequence 5′ to the variable region and the entire constant region containing both the secreted and membrane-bound 3′exons (Figure 1). A modified mouse H4 histone gene in the construct was not utilized in the present experiments, but will serve as an internal control gene in future studies. This pBR322-based plasmid DNA construct will be subsequently referred to as pμ.

Transfer of pμ DNA into Mice

Approximately 50 molecules of pμ DNA, linearized with Sal I, was microinjected into the male pronucleus of fertilized C57BL/6 mouse eggs, as previously described (Costantini and Lacy, 1982). A total of 284 microinjected eggs were transferred into the oviducts of nine pseudopregnant (CBA/J × C57BL/6J) F_1 female mice, either shortly after microinjection or after culture in vitro to the two-cell stage. Eight of the nine recipient females became pregnant, 23 of the embryos in these mice developed to term, and 13 of these survived to weaning.

When each of the 13 surviving mice was approximately four weeks old, a segment of tail was removed and DNA was extracted and analyzed for the presence of the microinjected gene. Tail DNA from five of the mice was found to contain the foreign gene. DNA samples were digested with Pst I (see Figure 1), fractionated by gel electrophoresis, transferred to nitrocellulose paper and probed with ^{32}P-labeled pBR322 DNA; Ig gene sequences were not included in the probe because these would cross-hybridize with endogenous mouse Ig genes. Three of the first eight mice that were screened contained DNA sequences hybridizing with pBR322 DNA (Figure 2). The intense 2.9 kb band in each positive lane (c, e, and h) corresponds with an expected Pst I fragment of pμ DNA (see Figure 1). In addition, each of the DNAs of the three positive mice—M52, M54, and M57—showed a band at 0.85 kb. This band corresponds in size to the other pBR322-containing Pst I fragment of circular pμ DNA, but is about 100 bp larger than the 0.75 kb Pst I–Sal I fragment generated by Pst I digestion of the Sal I linearized pμ DNA used for microinjection (Figure 2, lane j). The presence of the 0.85 kb fragment in the DNAs of the positive mice suggested that the ends of the linear DNA molecules became joined in a head-to-tail orientation after microinjection. Eco RI

Figure 2. Detection of $p\mu$ Sequences in Mouse DNAs

Mouse tail DNAs were digested with Pst I, separated by electrophoresis through a 0.7% agarose gel, transferred to nitrocellulose and hybridized with ^{32}P-labeled plasmid pBR322 DNA. Lanes M, ^{32}P-labeled molecular weight markers with the sizes indicated. Lanes a and j, 30 and 300 pg, respectively, of plasmid $p\mu$ DNA digested with Pst I and Sal I. Lanes b through i, approximately 10 μg of tail DNA from mice M51 through M58. The three mice containing $p\mu$ sequences are M52, M54, and M57.

digestion of the transgenic mouse DNAs confirmed this interpretation: in each case digestion with this enzyme produced a 6 kb fragment that hybridized to pBR322 DNA (data not shown). This fragment is predicted from the circular map of $p\mu$ DNA (Figure 1). When undigested mouse DNAs were similarly analyzed, the pBR322 sequences migrated with high molecular weight DNA (data not shown). These data, together with the pattern of germ line transmission observed (see below), suggest that the injected $p\mu$ DNA has integrated into the mouse genome in tandem arrays, as has been frequently observed with other microinjected genes (Costantini and Lacy, 1981; Brinster et al., 1981). The same DNA analysis was applied to the other five mice, two of which, M94 and M95, were also found to contain the microinjected μ gene (data not shown).

The number of copies of $p\mu$ DNA in the genome of each transgenic mouse was estimated by preparing dot blots with varying amounts of the mouse DNAs and of cloned $p\mu$ DNA, hybridizing with ^{32}P-labeled pBR322 DNA and quantitating the amount of hybridization by liquid scintillation counting. The number of gene copies per diploid mouse genome ranged from about 20 to 140 (Table 1).

The five transgenic mice were mated with normal C57BL/6 mice and tail DNAs from progeny were tested by dot blot hybridization to determine which had inherited the foreign $p\mu$ sequences. Twenty-seven out of 61 progeny from the male mouse M54, and 18 out of 40 progeny from male M95 contained DNA sequences that hybridized with the pBR322 probe. This frequency of transmission to progeny is consistent with the hypothesis that the foreign DNA was integrated at one site in the germ line DNA of

Table 1. $p\mu$ DNA and Serum Immunoglobulin Levels in Transgenic Mice

Mouse	$p\mu$ DNA Copy Number	Serum NIP Binding (μg/ml)		Heavy Chain Isotypes	
		λ	κ	μ	γ
M52	140	175.0	459	0.8	1.0
M54	30	91.0	432	0.9	0.9
M57	80	50.0	595	1.4	1.2
M94	68	0.8	54	1.2	1.1
M95	17	6.5	173	0.7	1.0
BL/6	0	0.5	108	1.0	1.0

DNA copy numbers of the μ NP^{α} gene per diploid genome were determined by quantitative dot blots. Transgenic mouse serum levels of NIP binding antibody with lambda (λ) or kappa (κ) light chains are expressed in μg/ml. Relative serum levels of IgM (μ) or IgG (γ) were determined by competitive radioimmunoassays and normalized to the value for C57BL/6 (BL/6).

these animals. Female M52 has so far transmitted the $p\mu$ sequences to 12 out of 15 progeny, which might reflect the presence of the foreign DNA on more than one chromosome. Female M57 did not produce any live offspring, and died at seven months of age. Female M94 has not transmitted the gene to any of her 26 offspring tested thus far, suggesting the possibility of extreme mosaicism. A mosaic distribution of the microinjected genes has been observed occasionally by others (Costantini and Lacy, 1981; Wagner et al., 1983; Palmiter et al., 1984).

Transcription of the Microinjected μ Gene

Spleen and liver tissue was obtained from transgenic mice M52, M54, and M57 by partial splenectomy and hepatectomy. Total RNA was isolated, size-fractionated by electrophoresis, transferred to nitrocellulose paper, and analyzed for the presence of transcripts from the microinjected sequences using a specific ^{32}P-labeled DNA probe. The probe was a cloned 220 bp Pst I fragment of the V_H17.2.25 gene segment (see Figure 1). A 2.4 kb transcript corresponding in size to the secreted form of μ mRNA was detected in the spleen RNA from the transgenic mice (Figure 3, lanes f–h). The 2.7 kb membrane-bound form of the μ mRNA could not be detected. This is in agreement with the previous demonstration that total spleen RNA contains only minor amounts of membrane-bound form of heavy chain transcripts (Alt et al., 1980a). The specific probe did not hybridize with spleen RNA from a normal C57BL/6 mouse (Figure 3, lane e), proving that the 2.4 kb transcript detected in the spleen RNA of the transgenic mice was derived from the introduced μ gene. To demonstrate the specificity of the DNA probe for V_H17.2.25 sequences under our hybridization conditions we hybridized the labeled probe to total RNA from myeloma MPC11 (lane i). Although the expressed V_H gene segment in MPC11 cells shares extensive sequence homologies with the V_H17.2.25 gene segment (Bothwell et al., 1981; Loh et al., 1983), no cross-hybridization of the heavy chain transcripts of MPC11 with the labeled DNA probe was observed. RNA isolated from the hybridoma 17.2.25 was

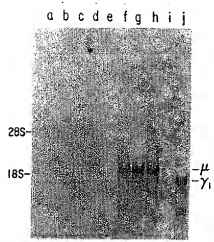


Figure 3. Size Analysis of $V_H17.2.25$ Specific Transcripts in Liver and Spleen RNA of Transgenic Mice

Ten micrograms of total formaldehyde-treated RNA was separated by electrophoresis through a 1% agarose gel in 6% formaldehyde, transferred to nitrocellulose paper and hybridized with a cloned ^{32}P nick-translated 220 bp PstI DNA fragment of the $V_H17.2.25$ segment. The sizes of the $V_H17.2.25$ transcripts were calculated from the position of the transcripts on the autoradiogram relative to the positions of 28S and 18S RNA on the ethidium bromide-stained gel. Lanes a–d were loaded with liver RNA, lanes e–h with spleen RNA. Lanes a and e contained RNA from a control C57BL/6 mouse, lanes b and f contained RNA from mouse M52, lanes c and g contained RNA from mouse M54 and lanes d and h contained RNA from mouse M57. Lane i was loaded with RNA from the myeloma MPC11 and lane j contained RNA from the hybridoma 17.2.25.

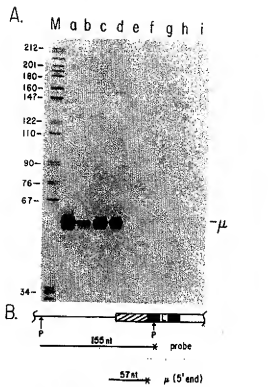


Figure 4. S1 Nuclease Analysis of $V_H17.2.25$ Specific RNA 5′ Ends of Spleen and Liver RNA

(A) Two micrograms of spleen RNA and 20 μg liver RNA from transgenic mice were hybridized to a single-stranded ^{32}P 5′ end-labeled DNA probe (see [B] for structure and size of probe) and digested with S1 nuclease as described in Experimental Procedures. The protected fragment was separated by gel electrophoresis and autoradiographed. Four micrograms of 17.2.25 hybridoma RNA was used for S1 nuclease mapping. Lane M, ^{32}P-labeled HpaII-digested pBR322 DNA. Lane a, RNA from from 17.2.25 hybridoma. Lanes b, c, and d, spleen RNA from transgenic mice M52, M54, and M57, respectively. Lanes f, g, h, liver RNA from mice M52, M54, and M57. Lanes e and i spleen and liver RNA from normal C57BL/6 mouse. The position of the 57 nucleotide long protected DNA fragment is indicated with μ. The bands migrating at 155 nucleotides correspond to RNA protecting the full length of the DNA probe.

(B) Structure of the leader region of the microinjected μ gene. The leader sequences (L) are drawn in black; intron sequences are drawn as open, and 5′ noncoding sequences are drawn as hatched bars. The structure and size of the DNA probe, which consists of a 155 bp PstI (P) DNA fragment, and that of the DNA fragment protected by the 57 5′ terminal nucleotides of the specific μ RNA are shown. The position of the ^{32}P label is indicated by a star.

included as a positive control and revealed the authentic 1.7 kb γ_1 mRNA (lane j). No hybridization of the labeled DNA probe could be detected in the lanes (b–d) loaded with liver RNA of the three transgenic mice. By inspection of the rRNA bands in the ethidium-bromide stained gel, the integrity and quantity of the liver RNA appeared to be the same as that of the spleen RNA (data not shown). The absence of specific μ transcripts in liver RNA was not due to a mosaic distribution of the introduced DNA in the transgenic mice because we confirmed the presence of the microinjected gene in both tissues of the animals by hybridization to ^{32}P-labeled pBR322 DNA (data not shown). These results demonstrate that the foreign μ gene is transcribed at a high level in the spleen but not in the liver of transgenic mice.

To confirm the tissue-specific expression of the transferred gene with a more sensitive assay and to examine the site of initiation of transcription we assayed spleen and liver RNA from the three transgenic mice by hybridization to a ^{32}P-labeled single-stranded DNA probe followed by S1 nuclease digestion and electrophoretic separation (Figure 4B). Spleen RNA of the transgenic mice protected an approximately 57 nucleotide long DNA fragment (Figure 4A, lanes b–d). The same protected fragment was generated by S1 nuclease treatment of hybridized 17.2.25 hybridoma RNA (lane a). Thus the identical initiation site of transcription is used in the introduced gene of the transgenic mice as in the endogenous gene of the hybridoma. A few specific μ transcripts were detected in the liver RNA of the transgenic mice (lanes f–h). The presence of μ transcripts in liver was probably due to the blood in this organ because we could decrease the number of specific μ transcripts to undetectable levels by perfusion of the transgenic liver prior to DNA isolation (see Figure 5A). No $V_H17.2.25$ specific sequences were detected in spleen or liver RNA from control C57BL/6 mice (Figure 4A, lanes e and i).

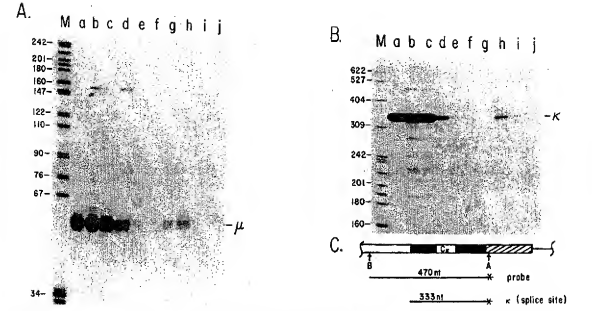


Figure 5. S1 Nuclease Analysis of V_H17.2.25 Specific and κ Specific Sequences in RNA from Various Tissues of the Transgenic Mouse M54-6

(A) Analysis of the 5′ ends of V_H17.2.25 specific μ transcripts. Ten micrograms of total RNA from the tissues was hybridized to the ^{32}P-labeled 155 nucleotide long Pvu II DNA fragment shown in Figure 4B. Four micrograms of 17.2.25 hybridoma RNA was used for hybridization. The position of the DNA fragments protected by the 5′ terminal sequences of V_H17.2.25 transcripts is marked with μ. Lane M, size marker (pBR322 DNA cleaved with Hpa II). Lane a, 17.2.25 hybridoma RNA. Lanes b through j, RNA from lymph nodes, spleen, thymus, kidney, brain, heart, lung, liver, fibroblasts.

(B) Analysis of κ transcripts. Five micrograms of total RNA was hybridized to the splice probe shown in Figure 5C. The position of the protected fragment is indicated with κ. Lane M, size marker. Lane a, RNA from myeloma MPC11 cells. Lanes b through j are numbered as in (A).

(C) Structure of the κ splice probe. The exon of the C_κ constant region is depicted as a black bar. Intron and 3′ noncoding sequences are drawn as open and hatched bars, respectively. The positions of the Ava II (A) and Bst NI (B) sites, used to generate the DNA probe, are shown.

The relative number of V_H17.2.25 specific transcripts in the spleens of the transgenic mice M52, M54, and M57 were determined by densitometric scanning of different exposures of the autoradiogram using transcription of the rearranged endogenous V_H17.2.25 gene in the hybridoma as standard. The number of specific μ transcripts in the spleen of mouse M54 was calculated to be the same as that in the 17.2.25 hybridoma. This hybridoma was determined to contain 2000 V_H17.2.25 specific RNA molecules per cell (unpublished results). The spleens of mice M52 and M57 accumulated 35% and 85% of the number of specific μ transcripts found in the hybridoma. Mouse M52, with the highest copy number of the introduced gene (140 copies), has a 3-fold lower RNA level in spleen cells compared with mouse M54 containing 30 foreign μ gene copies (see Table 1). Therefore the levels of transcription of the microinjected genes did not correlate with the number of gene copies integrated in the chromosomes.

Tissue Specificity of μ Gene Transcription

A more detailed study of the tissue specificity of μ gene expression was carried out using progeny of mice M54 and M95 to avoid the possibility of a mosaic distribution of the injected gene. Various tissues were collected from a six-week-old second generation mouse, M54-6, and total RNA was isolated. RNA from the tissues was analyzed by S1 nuclease mapping using a 5′ end-labeled specific DNA probe (Figure 5A). High levels of specific μ gene transcription were detected in all lymphatic tissues analyzed. Lymph node RNA contained twice as much V_H17.2.25 specific μ RNA as spleen (Figure 5A, lanes b and c). The level of specific μ gene transcription in thymus was determined to be about 35% of that in spleen (lane d).

The expression of the microinjected μ gene in lymph nodes was so high that any contamination of a tissue with lymph nodes would have resulted in detection of specific μ transcripts. Thymus preparations are known to be easily contaminated with parathymic lymph nodes. Although we attempted to avoid the parathymic nodes in the dissection, to measure B cell contamination we examined transcription of the endogenous κ light chain Ig gene, a gene whose transcription should be restricted to B cells and is active in about 95% of such cells. Because the 5′ ends of κ mRNAs differ from one another, we prepared a 5′ end-labeled DNA probe which spanned the boundary of the large J_κ/C_κ intron and the C_κ exon (Figure 5C). We used this labeled DNA probe for S1 nuclease mapping of the splice site at the 5′ end of the C_κ exon which is common to all κ mRNAs.

RNA from the tissues was hybridized with the "splice

probe," digested with S1 nuclease and the protected fragments were separated by electrophoresis. RNA from spleen and lymph nodes contained similar numbers of κ transcripts (Figure 5B, lanes b and c). As a positive control, RNA from cells of the myeloma MPC11 was used (lane a). The presence of κ gene transcripts in thymus (lane d), determined to be about 12% of the number in spleen, revealed contamination of the thymus preparation with κ-producing cells. This level of contamination with B cells, however, does not account for the high level of 17.2.25 gene expression observed in thymus (compare Figure 5A) indicating that the microinjected gene is probably active in thymocytes.

Nonlymphoid tissues were also analyzed for the presence of V_H17.2.25 specific μ RNA sequences. Heart and lung both contained μ transcripts (Figure 5A, lanes g and h) which represented about 5% and 8% of the number of specific μ RNA molecules detected in spleen. Kidney RNA contained about 2% of V_H17.2.25 specific transcripts present in spleen (lane e). No μ transcripts were detected in RNA from brain, perfused liver, or primary fibroblasts cells (lanes f, i, and j). To assess the question of whether the specific μ transcripts present in heart, lung, and kidney were due to transcription of the microinjected gene in these tissues or due to contaminating lymphoid cells in the blood of the tissues we again measured κ transcripts in the RNA preparation from these tissues. Transcripts containing C_κ sequences were found in the RNA from lung tissue, at about 5% of the level in spleen (Figure 5B, lane h). The extent of B cell contamination of lung thus could account for the μ transcripts found, suggesting that the microinjected gene was not transcriptionally active in this tissue. The presence of some κ transcripts in kidney RNA (Fig. 5B, lane e) indicated that kidney was contaminated with B cells and, again, that the microinjected gene was probably not transcribed. Heart was the only nonlymphatic tissue of the transgenic mouse M54-6 in which the microinjected gene appeared to be active because virtually no κ transcripts could be detected in RNA from that tissue (Figure 5B, lane g).

This tissue distribution of specific μ transcripts was confirmed by analysis of another second generation mouse, M54-3, and similar results were also obtained with a progeny from transgenic mouse M95 (data not shown).

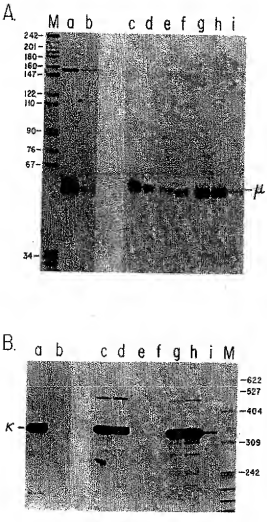
Cell-Type Specificity of Transcription

To investigate the expression of the microinjected μ gene in individual lymphoid and nonlymphoid hematopoietic cell types, a number of fractionation procedures were used. To study macrophages, which belong to the myeloid lineage, peritoneal cells were isolated from a second generation mouse, M54-48, previously injected with thioglycolate to increase the number of macrophages (Galilly and Feldman, 1967). The cells were incubated in a tissue culture plate to allow macrophages to attach. Phagocytosis of latex particles was used to show that virtually all of the adherent cells were macrophages. We isolated total RNA from the cells and assayed for the presence of V_H17.2.25 sequences by S1 nuclease mapping of the specific mRNA 5′ ends. Very few specific μ transcripts were detected in the RNA from macrophages (Figure 6A, lane b), about 2% of that found in total lymph node RNA from the same animal (lane a). We could not detect any κ transcripts in the RNA from macrophages (Figure 6B, lane b) indicating that the low level of specific μ transcripts in macrophages was not due to contaminating B cells.

For the purification of B and T cells we isolated spleen and mesenteric lymph nodes from second generation transgenic mice M54-62 and M54-64. Cell suspensions were prepared and surface Ig-positive B cells were purified by binding to plastic dishes coated with rabbit anti-mouse Ig. To reduce the background of nonspecific binding of cells because of their F_c receptor, we used $F(ab')_2$ fragments of the anti-mouse Ig antibody. The bound cells were washed, RNA was isolated and analyzed by S1 nuclease mapping. V_H17.2.25 specific μ transcripts were detected in the RNA from B cells of spleen and lymph nodes (Figure 6A, lanes c and d), proving that the microinjected μ gene is expressed in surface Ig-positive B cells. To purify T cells, cell suspensions from lymph nodes and thymus were first depleted of surface Ig-positive B cells by binding to plastic dishes coated with rabbit anti-mouse Ig. Unbound cells were then transfered to plastic dishes coated with the $F(ab')_2$ fragment of anti-Lyt 2 antibody. Surface Lyt 2-positive T cells, which should represent 50% of all mature T cells present in lymph nodes and 90% of thymocytes, were bound and RNA was isolated. Analysis of the RNA revealed the presence of specific μ transcripts in mature T cells from lymph node and thymus (lanes e and f). The number of specific μ transcripts in T cells from lymph nodes was only about 2-fold lower than that in B cells from lymph nodes, indicating that the microinjected gene is almost equally active in mature B and T cells.

These cell populations probably contribute most of the specific μ signal seen with total RNA from whole spleen or lymph nodes, because the cells remaining unbound after the above described fractionations did not show a significantly higher level of V_H17.2.25 specific transcripts (Figure 6A, lanes g–i).

To demonstrate the purity of the B and T cell preparations, we measured κ transcripts in the RNA. Large amounts of κ mRNA were detected in B cell RNA from spleen and lymph nodes, as expected (Figure 6B, lane c and d). Virtually no κ transcripts were found in T cell RNA from lymph node and thymus (lanes e and f), indicating that the T cell preparations were more than 96% free of contaminating B cells. This result verified the observation that Lyt 2-positive T cells from lymph nodes and thymus indeed expressed the microinjected μ gene. Because the cells went through some manipulations prior to RNA isolation we wanted to control for the quantity and integrity of the RNA of each cell preparation. We determined the presence of beta-2 microglobulin transcripts in each RNA preparation by S1 nuclease mapping of the splice site of the second exon of the gene (Figure 6D; Parnes and Seidman, 1982). Similar numbers of beta-2 microglobulin



transcripts were detected in B cells of spleen and lymph nodes (Figure 6C, lanes c and d) and in T cells from lymph nodes (lane e). Fewer beta-2 microglobulin transcripts were found in T cells from thymus (lane f).

Formation of Functional Antibody by Combination of the Specific μ Heavy Chain with Endogenous λ1 Light Chains

The synthesis of a functional μ 17.2.25 polypeptide was monitored by measuring the formation of NP binding antibody, composed of the specific μ heavy chains and endogenous λ1 light chains. We tested the sera of the transgenic mice for elevated levels of NP binding antibody by a standard radioimmunoassay using the derivative 5-iodo-NP (NIP). NIP–BSA coated wells were incubated with serial dilutions of serum, and bound immunoglobulins were detected by binding of ^{125}I-labeled rabbit anti-mouse λ antibody. A dramatic increase in the level of NP binding antibody with λ light chain specificity was observed for sera from mice M52, M54, M57, and M95 varying from 5 to 175 μg/ml in concentration (Table 1). Normal C57BL/6 mice and negative littermates that did not possess the specific μ gene were negative for serum NP binding activity, giving the same low level of 0.5 μg/ml. Mouse M94 serum contained only 2-fold more NP binding antibody with λ specificity than normal serum, although the με DNA copy number in its tail was 68 (Table 1). The NP binding antibodies of all transgenic mice have λ1 light chains because a ^{125}I-labeled rat anti-mouse λ1-specific monoclonal antibody, LS136, showed the same level of NP binding as the polyclonal anti-lambda antibody (data not shown).

The serum of transgenic mice showed only a 2- to 3-fold increase in the concentration of κ-specific NP binding antibody, as determined by binding of ^{125}I-labeled rat anti-mouse κ monoclonal antibody, 187.1 (Table 1). The small increase of κ-containing antibody might indicate that the

Figure 6. S1 Nuclease Analysis of V_H17.2.25, κ, and Beta 2 Microglobulin Sequences in Total RNA from Transgenic Macrophages, B and T Cells.
(A) Analysis of the 5′ ends of V_H17.2.25 specific μ transcripts. Two micrograms of total RNA from various cells was hybridized to the μ 5′ end probe shown in Figure 4B. Lane M, ^{32}P-labeled size marker (pBR322 DNA cleaved with Hpa II). Lanes a and b, RNA from lymph nodes and purified macrophages of the transgenic mouse M54-46. Lanes c and d, RNA from surface Ig-positive B cells from spleen and lymph node, respectively. Lanes e and f, RNA from Lyt 2-positive T cells from lymph nodes and thymus. Lanes g through i, RNA from spleen, lymph node, and thymus cells which had been previously depleted of surface Ig- and Lyt 2-positive cells.
(B) Analysis of κ transcripts. Two micrograms of total RNA was hybridized to the κ splice probe shown in Figure 5C. The position of protected fragments is indicated with κ. The lanes are numbered as in (A).
(C) Analysis of beta 2 microglobulin transcripts. Two micrograms of total RNA was used for S1 nuclease mapping with a double-stranded beta 2 splice probe depicted in (D). The lanes are numbered as in (A). The 203 nucleotide long protected fragment is indicated with β_2. The 235 nucleotide long fragment represents the renatured full-length DNA probe.
(D) Structure and size of the beta-2 microglobulin probe. The probe consists of an Eco RI/Sac I DNA fragment, 5′ end labeled at the Eco RI site. The probe contains sequences of the second exon of the gene (black bar) and intron sequences (open bar).

μNP^b 17.2.25 heavy chain is capable of forming a functional NP-specific antibody when combined with a few kappa light chains, but the minimal increase is in agreement with the observation that NP-binding antibody produced in the primary immune response in BALB/c mice contains predominantly λ1 light chains (White-Scharf and Imanishi-Kari, 1981).

The NP-binding antibody in transgenic mice contained μ heavy chains because only anti-μ, and not anti-IgG1, IgG2a, or IgG2b antisera, detected the increased level of NP binding (data not shown). The progeny of M54 and M95 were screened for λ-specific NP binding activity. Positive littermates that inherited DNA sequences were also positive for NP binding activity. In addition, positive littermates had a level of λ-specific NP binding antibody comparable to their transgenic parent. Although we cannot yet rule out that the transgenic mice have shown elevated expression of the endogenous NP^b gene, it is much more likely that the high constitutive serum level of NP-specific antibody in the transgenic mice results from a combination of the μ heavy chain encoded by the microinjected gene with endogenous λ1 light chains.

The microinjected rearranged heavy chain gene was present from the earliest developmental stages of the transgenic mice. Thus expression of the gene and/or functional antibody might have altered normal immunoglobulin production. As a crude measure we assayed the sera from transgenic mice and their negative littermates for the presence of total μ or γ heavy chains, and kappa or lambda light chains, by competitive radioimmunoassay. Transgenic mouse serum competed as well as normal mouse serum with ^{125}I-labeled IgM, IgG, or kappa antibodies for anti-IgM, IgG, or kappa binding (Table 1). A modest 2-fold increase in lambda containing immunoglobulins was detected in serum from mouse M54. Thus the expression of a microinjected heavy chain gene had no obvious effect on the levels of endogenous serum immunoglobulins.

Discussion

High Level of Expression of the Microinjected μ Gene

The expression of the microinjected rearranged μ heavy chain gene in transgenic mice demonstrates that DNA sequences that are required for the transcriptional activation of the gene during B cell ontogeny are located within the introduced DNA construct. The injected Ig gene was found to be expressed at high levels in four out of five transgenic mice. The one exception, mouse M94, had the gene integrated in chromosomal DNA of the tail but revealed only a 2-fold elevation of NP binding antibody in the serum (Table 1). Because this mouse has not transmitted the microinjected gene to any of her 26 progeny so far, we suspect that the lack of NP binding antibody in the serum was due to a mosaic distribution of the injected μ gene in spleen and germ line, rather than to a transcriptional inactivity of the gene. A similarly high frequency of expression has been reported by Brinster et al. (1983) who introduced a rearranged κ light chain gene into the mouse germ line.

These results contrast markedly with those obtained for several other introduced genes, which are expressed in a lower proportion of transgenic mice or at much more variable levels, presumably because of the influence of chromosomal position (Jaenisch et al., 1981; Wagner et al., 1981; Brinster et al., 1981; Lacy et al., 1983; Palmiter et al., 1983). The question that arises is why microinjected immunoglobulin genes appear to behave differently, showing only slight variation of expression levels between different transgenic mouse lines. It is possible that the *cis*-acting transcriptional enhancer elements associated with immunoglobulin genes (Gillies et al., 1983; Banerji et al., 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984) may be capable of counteracting negative chromosomal position effects.

The Foreign μ Gene is Regulated in a Tissue-Specific Fashion in Transgenic Mice

Transgenic mice are ideal for the study of tissue specificity of gene expression because all cells should contain the same number of copies of the introduced gene in the identical chromosomal location. Therefore we can compare directly the level of transcription in one tissue with that in another tissue. Our results demonstrate that expression of the microinjected Ig μ gene is tissue-specific. High levels of transcription can be detected in lymphoid tissues, while nonlymphoid tissues, with the exception of heart, contain virtually no specific μ transcripts.

Because the functionally rearranged foreign heavy chain gene is present in cells that do not produce the specific μ transcripts, we can conclude that DNA rearrangement, which occurs only in cells of the B cell lineage, does not solely account for the lymphoid tissue specificity of μ gene expression. Cis-acting DNA sequences residing in the introduced rearranged μ gene must control its selective transcription. A similar conclusion was reached by Brinster et al. (1983), who showed that an introduced rearranged light chain gene was expressed in spleen but not in liver. Both sets of data support DNA transfection experiments which showed that rearranged Ig genes are transcriptionally inactive in fibroblast cells (Gillies et al., 1983; Stafford and Queen, 1983). The heavy chain gene enhancer was demonstrated to be inoperative in fibroblast cells, leading to the hypothesis that this regulatory element determines the tissue specificity of Ig gene expression (Gillies et al., 1983; Banerji et al., 1983). More recent studies in which the heavy chain gene enhancer was replaced with a nonspecific viral enhancer, however, have demonstrated that other regulatory elements are involved in determining tissue specificity of μ gene transcription (R. Grosschedl and D. Baltimore, unpublished data).

Although expression of the microinjected μ gene was generally restricted to lymphoid tissues, the gene appeared to be transcribed at a low level in heart for reasons that are unclear. One possible explanation is that cardiac mus-

cle cells might contain *trans*-acting factors which can partially substitute for lymphoid cell-specific regulatory factors interacting with regulatory DNA sequences. Heart and hematopoietic cells are derived from common progenitor cells during development and this fact might account for μ gene expression in heart. Alternatively, the μ genes might be integrated in a chromosomal locus that is activated in heart. This possibility appears unlikely because mouse lines M54 and M95, in which the μ genes are presumably integrated at different chromosomal positions, both show μ gene transcription in heart.

Macrophages, which are terminally differentiated nonlymphoid hematopoietic cells, showed a very low but detectable level of specific μ gene transcription, about 2% of that in spleen. The macrophage preparation was determined to be free of B cells but may have been contaminated with some T cells. Because the μ gene is expressed in T cells at high levels, even a *minimal* contamination could account for the few specific μ transcripts. Previous analysis of RNA from a macrophage cell line showed that the endogenous μ genes seem to be inactive (Kemp et al., 1980) and our result is consistent with this observation. In contrast, tumor cells representing early stages of the myeloid lineage contain aberrant or "sterile" μ transcripts derived from the unrearranged μ constant region at a level similar to that found in B cell lymphomas (Kemp et al., 1980). This implies that these immature myeloid cells contain factors that can interact with *cis*-acting elements associated with the heavy chain genes. It remains to be established whether in transgenic mice, cells representing earlier stages of the myeloid lineage transcribe the microinjected μ gene at a higher level than terminally differentiated macrophages.

The Rearranged Gene is Expressed in Both B and T Cells

The most interesting result we obtained concerning expression of the foreign μ gene in transgenic mice was the observation that the gene is almost equally active in purified B and T cells. Surface Ig-positive B cells of mouse M54 synthesize as many V_H17.2.25 specific transcripts as hybridoma 17.2.25 cells. The level of transcription of the microinjected μ gene in Lyt 2-positive T cells is only 2-fold lower than that in B cells. This result implies that T cells contain probably all *trans*-acting factors required for high level expression of rearranged Ig heavy chain genes. Previous analysis of RNA from various T cell lines (Kemp et al., 1980; Alt et al., 1982) and whole thymus (Alt et al., 1982) revealed the presence of "sterile" μ transcripts. The synthesis of such sterile μ transcripts might reflect an active enhancer element because in some cases the 5′ ends of sterile μ transcripts have been mapped to a pseudopromoter (Nelson et al., 1983) located close to the enhancer (Gillies et al., 1983; Banerji et al., 1983). From our data we can conclude that not only the enhancer but also all other *cis*-acting elements required for high level μ gene transcription are active and operate properly in Lyt 2-positive T cells.

The high level expression of the rearranged μ gene in T cells contrasts with the normally exclusive pattern of B and T cell receptor gene expression. In normal development, functional rearrangement of the B cell receptor genes (or Ig genes) is restricted to B lymphocytes whereas rearrangement of the T cell receptor genes occurs in T cells (Tonegawa, 1983; Hedrick et al., 1984a, 1984b; Yanagi et al., 1984). Our experiments suggest that control of gene rearrangement may be the only mechanism that determines the specificity of heavy chain expression within the lymphoid cell lineage. Similarly, the cell specificity of T cell receptor expression may also be controlled at the level of DNA rearrangement. It will be very interesting to learn whether genes for B and T cell receptors utilize common *trans*-acting factors for transcriptional regulation. In contrast, the tissue-specific expression of heavy chain genes in lymphoid as opposed to nonlymphoid cells does not depend on the tissue specificity of DNA rearrangement, but is determined by the interaction of lymphoid-specific factors with *cis*-acting regulatory elements.

Expression of the Microinjected μ Gene Does Not Affect the Serum Levels of Endogenous Immunoglobulins

Expression of the foreign μ gene in B cells of transgenic mice generated NP binding antibody when the specific heavy chain associated with endogenous λ1 light chains. The serum levels of NP binding antibody in transgenic mice was stimulated up to 400-fold over that in normal C57BL/6 mice (Table 1). This assay probably underestimates the number of specific μ heavy chains synthesized, because we monitor only those that have associated with endogenous λ1 light chains.

All transgenic mice had normal serum levels of the endogenous immunoglobulins, suggesting that expression of the foreign μ gene did not completely prevent rearrangement and expression of endogenous Ig genes. The mechanism which ensures that only a single heavy chain gene and a single light chain gene will be functionally rearranged during B cell differentiation ("allelic exclusion") may involve a feedback mechanism in which the synthesis of an Ig heavy or light chain polypeptide blocks any further rearrangements of heavy or light chain genes, respectively (Alt et al., 1980b, 1981; Coleclough et al., 1981). We do not yet know whether transgenic B cells express simultaneously the foreign μ gene and endogenous heavy chain genes but the transgenic mice offer us the opportunity to study this aspect of immunoregulation in detail.

Experimental Procedures

Construction of $p\mu$

pLV2 DNA, which has an Eco RI DNA fragment containing the functionally rearranged V_HDJ_H gene segment of the hybridoma 17.2.25 and flanking sequences linked to the genomic Eco RI/Bam I DNA fragment containing the C_μ constant region (D. Loh and C. Queen, unpublished data), was partially cleaved with Eco RI. The Eco RI site 5′ to the V_HDJ_H gene segment was converted to a Sal I site by blunting the 5′ ends and by addition of Sal I linkers. A pBR322 derivative plasmid, lacking the "poison" sequences between nucleotides 1120 and 2490 was obtained by changing the Eco RI

site in pML1 (Lusky and Botchan, 1981) to a Sal I site. Furthermore, the Nru I site of the plasmid at nucleotide position 973 was converted to a Hind III site. Vector DNA was digested with Sal I and Hind III and ligated with the Sal I/Xho I DNA fragment comprising the pT17.2.25 gene and an Xho I/Hind III DNA fragment containing a modified mouse H4 histone gene (R. Groschedl and D. Baltimore, unpublished data). The resulting clone, with the Sal I site of the μ gene fused to the Sal I site of the vector, was called pμ.

DNA injections into Mouse Eggs

pμ DNA was linearized with Sal I and the single-stranded ends were made double-stranded with DNA polymerase (Klenow fragment) and dNTPs. Fertilized one-cell eggs were recovered from the oviducts of C57BL/6J female mice that had mated with C57BL/6J males the previous night. Approximately 1 pl of the linearized pμ DNA at a concentration of 0.8 μg/ml (or 50 molecules/pl) was microinjected into one pronucleus of each egg as previously described (Costantini and Lacy, 1982).

Isolation of DNA

For the isolation of tail DNA, approximately one-third of the tail was cut off, the bone was removed and discarded, and the skin was weighed (the final yield of DNA was 2–3 μg per mg of tail skin). The skin was then incubated overnight at 55°C in 1.0 ml of 0.1 M EDTA, 0.05 M Tris-HCl (pH 8), 0.5% sodium dodecyl sulfate, 500 μg/ml proteinase K, on a rocking platform; no physical disruption was necessary. The resulting homogenate was centrifuged to remove undigested hair, extracted once with phenol and once with phenol/chloroform. The DNA was precipitated by addition of sodium acetate pH 6.0 to 0.3 M and one volume of ethanol, at room temperature. The DNA pellet was washed once with 70% ethanol, dried and resuspended in 10 mM Tris-HCl (pH 8), 1 mM EDTA. Restriction digestions and DNA/DNA hybridization analyses were performed as previously described (Costantini and Lacy, 1982) except that RNAase A (5 μg/ml) was included in the restriction digests to digest residual RNA contaminating the tail DNA samples.

Isolation and Purification of RNA

Total RNA was prepared from the various cells and tissues by the guanidinium isothiocyonate extraction procedure (Chirgwin et al., 1979) and purified on CsCl gradients (Glisin et al., 1974).

Size Analysis of $V_H17.2.25$ Specific RNA

Ten micrograms of total RNA was treated with 6% formaldehyde and fractionated by electrophoresis through a 1% agarose gel in 6% formaldehyde, 20 mM phosphate buffer (pH 7.0) (Lehrach et al., 1977) at 100 V for 5 hr. After staining the gel with ethidium bromide and destaining, the RNA was transferred to nitrocellulose paper and hybridized with a ^{32}P nick-translated, cloned $V_H17.2.25$ specific Pst I DNA fragment as described by Alwine et al. (1977).

Perfusion of Liver

Liver was perfused as described (Leffert et al., 1979). The tissue was disaggregated by collagenase treatment and hepatocytes were enriched by centrifugation.

Preparation of Cell Populations

Primary fibroblast cells were obtained from muscular fascia. Fibroblasts were grown in DME supplemented with 15% fetal calf serum. Macrophages were prepared from 10^7 peritoneal cells of one thioglycolate-stimulated (Gallily and Feldman, 1967) transgenic mouse. Cells were collected, washed, and transferred onto a 10 cm tissue culture plate containing RPMI with 10% fetal calf serum and 50 μM 2-Mercaptoethanol. After 5 hr of incubation nonadherent cells were removed. One micrometer latex particles were added to a portion of the adherent cells to identify the cells as macrophages by uptake of the latex particles. B cells and T cells were purified from spleen and mesenteric lymph nodes by binding of the cells to plastic dishes coated with $F(ab')_2$ anti-Ig and $F(ab')_2$ anti-Lyt 2, respectively, as described (Mage et al., 1977; Wysocki and Sato, 1978; N. Landau, unpublished results).

S1 Nuclease Analysis

From 2 to 20 μl total RNA was hybridized in 10 μl hybridization buffer (Hentschel et al., 1980) with ^{32}P 5′-end-labeled DNA fragments. The hybridization temperatures were 38°C for the μ DNA probe and 50°C for the κ and beta-2 microglobulin DNA probes. The hybrids were digested with 50 U of S1 nuclease (PL–Biochemicals) at 37°C for 1 hr. The protected DNA fragments were separated by electrophoresis through 8% polyacrylamide-urea gels.

Immunoglobulin Radioimmunoassays

NP binding activity assays and immunoglobulin isotype competitive binding assays were conducted on plastic microtiter wells according to White-Scharf and Imanishi-Kari (1982). An NP derivative, NP (4-hydroxy-5-iodo-3 nitrophenyl)acetyl, coupled to bovine serum albumin (BSA), was provided by Dr. Thereza Imanishi-Kari. In direct binding experiments, wells were coated with 5 μg/ml NP-BSA after which free protein binding sites were blocked with 1% BSA. Serum dilutions were incubated in the wells and subsequently washed with 150 mM NaCl. Wells were then incubated with ^{125}I-antibody radiolabeled by the chloramine-T method (Greenwood et al., 1963), and ^{125}I dpm counted after extensive 150 mM NaCl washes. Polyclonal rabbit anti-mouse λ_1, rat anti-mouse μ, or monoclonal antibodies LS136 (anti-mouse λ_1) or 187.1 (anti-mouse ϵ) were iodinated. Serum concentrations of NP binding antibody were estimated by a comparison with purified anti-NP antibody 124/57 (μ, λ_1) on 18.85 (λ_{as}, ϵ). Similarly, wells coated with 10 μg/ml anti-BALB/c 17.2.25 immunoglobulin were treated with serum dilutions, and probed with ^{125}I rat anti-mouse μ for NP^a-specific binding. For the isotype competition experiments, wells were coated with 10 μg/ml polyclonal rat anti-mouse μ, anti-mouse γ_1, anti-mouse γ_{2a}, or anti-mouse γ_{2b} antibodies, and free protein binding sites blocked with 1% BSA. Then serial serum dilutions were incubated with an equal volume of ^{125}I-labeled specific mouse immunoglobulin: P1.37.9(IgM), B6.63 (IgG$_1$), P8.44.18 (IgG$_{3a}$), B6-1E (IgG$_{3b}$), or a mixture of the IgG antibodies on the coated wells. Wells were then washed extensively with 150 mM NaCl before scintillation counting.

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Immunoglobulin gene transcription ceases upon deletion of a distant enhancer

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The tissue-specific Eμ enhancer within the immunoglobulin heavy chain (IgH) locus has recently been shown to be essential for efficient V region gene assembly in early B lineage cells. However, we and others have shown that late stage, Ig-secreting cells can produce IgH in the absence of Eμ. In the present study we have explored the notion that another enhancer found in the far 3′ region of the IgH locus (3′αE) takes on an important regulatory role in cells that have reached this terminal stage in B cell development. The technique of homologous recombination was used to disrupt the 3′αE region in an Eμ-deficient, Igγ2a-secreting cell line. Loss of 3′αE completely abolished Ig heavy chain gene expression, demonstrating that transcription of this gene was dependent upon sequences that reside over 70 kb downstream. The ability of these sequences to function efficiently in the absence of Eμ may also provide an explanation for deregulated c-*myc* expression in many Ig-secreting tumors.

Keywords: 3′αE/Eμ/homologous recombination/c-*myc*/myeloma

Introduction

Immunoglobulin heavy and light chains genes are unlike most other tissue-restricted genes in that they must be assembled from dispersed gene segments. The assembly of these segments (V and J for Ig light chain, V, D and J for Ig heavy chain) is accomplished through a series of DNA recombination events. V–D–J joining in the immunoglobulin heavy chain (IgH) locus results in the formation of a μ heavy chain transcription unit. After Ig light chain gene assembly has also taken place, the developing B lymphocyte begins to display the characteristic IgM molecule on its cell surface, where it serves as an antigen receptor. Antigen- and T lymphocyte-driven events can induce an IgM-positive cell to switch to expression of another class of heavy chain, but with the same antigen specificity. This switch is accomplished through a second type of DNA recombination event, the 'Ig heavy chain class switch' (reviewed in Max, 1993).

Within the non-coding sequences separating the V_H and C_H regions of an assembled μ heavy chain gene lies a transcriptional enhancer, Eμ. Eμ was one of the first non-viral transcriptional enhancers to be described and was the first example of a tissue-specific enhancer (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983). While initially identified by virtue of its effects on IgH gene transcription, Eμ has since been shown to enhance the V–D–J recombination process as well (Chen *et al.*, 1993; Serwe and Sablitzky, 1993). This, together with the early discovery that the IgH genes of Ig-secreting cells (a late stage in B lymphocyte development) could function without Eμ (Klein *et al.*, 1984; Wabl and Burrows, 1984; Aguilera *et al.*, 1985; Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985), led to the notion that Eμ might serve an important function in early stage B cells, but a less critical one in later stage cells.

One of the first Ig-secreting cell lines shown to secrete Ig in the absence of Eμ was the murine myeloma 9921 (Eckhardt and Birshtein, 1985; Zaller and Eckhardt, 1985). 9921 produces copious amounts of IgG2a. Eμ was lost from the expressed γ2a gene of 9921 as the result of a heavy chain class switch (see Figure 1). Additional myeloma lines that lacked Eμ were discovered in a number of laboratories, demonstrating that Eμ-independent gene expression was not the peculiar attribute of a single cell line (Klein *et al.*, 1984; Wabl and Burrows, 1984; Aguilera *et al.*, 1985; Klein *et al.*, 1985). While loss of Eμ is not a requisite step in the differentiation of B lymphocytes (the heavy chain class switch usually takes place without loss of Eμ), the demonstration that IgH genes could function without this enhancer suggested that transcriptional control of these genes was more complex than initially appreciated. As a result, several laboratories began a search for additional enhancers within the IgH locus.

Coinciding with the discovery of Eμ-independent IgH gene expression were descriptions of chromosomal translocations between the IgH locus and the oncogene c-*myc*. Such chromosome translocations are characteristic of both Burkitt's lymphoma (humans) and mouse myeloma (reviewed in Klein and Klein, 1985) and generally lead to deregulated expression of c-*myc*. Furthermore, cell fusion studies showed that the translocated c-*myc* was being expressed in a B cell-specific manner (Greenberg *et al.*, 1989). It was suggested by several groups that juxtaposition of c-*myc* with a B cell-specific control element(s) within the IgH locus was the explanation for abnormal c-*myc* expression in these tumor lines. Although Eμ was initially considered a good candidate, it was subsequently noted that in many mouse myelomas the translocated c-*myc* and Eμ lay on opposite translocation products (reviewed in Cory, 1986). Again, there was an impetus to search for additional IgH locus enhancers.

A few years ago a second DNA segment with transcriptional enhancer activity was identified at the 3′-end of the murine IgH locus (Dariavach *et al.*, 1991; Lieberson *et al.*, 1991). This enhancer region, 3′αE, lies ~200 kb 3′ of the

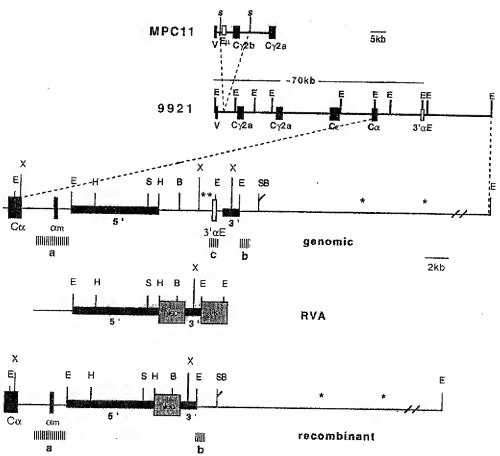

Fig. 1. Targeted deletion of the 3′αE region in 9921. MPC11, a γ2b-secreting cell line, gave rise to 9921 through an Ig heavy chain class switch. Relevant regions of the IgH loci in these two cell lines are shown (MPC11 and 9921 maps respectively). The break points of the class switch recombination event are indicated (S). The position of the intronic IgH enhancer Eμ is indicated by an open box, as is the 3′ region enhancer (3′αE). The V_H region expressed in MPC11 and 9921 is shown as a filled box (V); constant region coding sequences are also shown as filled boxes (Cγ2b, Cγ2a, Cε, Cα). *Eco*RI restriction sites are indicated (E) on the 9921 locus map. The distance between 3′αE and the expressed V_H is >70 kb. Below the MPC11 and 9921 locus maps is a detail of the region 3′ of Cα in 9921 (genomic), a map of the replacement targeting vector (RVA) and the predicted structure of the genomic locus after homologous recombination with RVA (recombinant). Thick lines designated 5′ and 3′ refer to regions of homology between RVA and genomic DNA. The Cα membrane exon is included in the three maps (αm) and 3′αE is designated as an open box. Specific restriction sites which flank 3′αE are indicated and include *Bam*HI (B), *Eco*RI (E), *Sac*I (S), *Xmn*I (X) and *Hind*III (H). Except for *Eco*RI, the map does not include all of the restriction sites for these enzymes. Asterisks indicate the positions of four DNase I hypersensitive sites identified by others (Giannini *et al.*, 1993; Madisen and Groudine, 1994). *pgk–neo*^{r} sequences and the MC1-tk gene are shown as shaded boxes (Neo and tk respectively). The HSV-tk gene was included, but not used, in the RVA vector as a means for selecting against cells that had randomly integrated this vector (see Materials and methods). The probes used for Southern blot analysis are represented by vertically striped bars underneath the genomic configurations and are given letter designations (a, b and c).

J_H gene segments where V_H gene assembly takes place. As measured in transient transfection assays, 3′αE was found to be less powerful than Eμ in some Ig-secreting cell lines, but equivalent to Eμ in others (Dariavach *et al.*, 1991; Lieberson *et al.*, 1991; Singh and Birshtein, 1993). 3′αE was, at best, a weak transcriptional enhancer in pre-B and surface Ig$^+$ cell lines (Dariavach *et al.*, 1991; Singh and Birshtein, 1993; Madisen and Groudine, 1994) and a recent study of transgenic mice carrying a 3′αE-controlled reporter gene supports the notion that this element begins to function only after B cells are activated (Arulampalam *et al.*, 1994). Interestingly, analyses of mice in which B lineage lymphocytes lack 3′αE have suggested that 3′αE plays a role in regulating the Ig heavy chain class switch (Cogne *et al.*, 1994). The emerging impression is that 3′αE and Eμ provide essential functions at separate times during B lymphocyte development. Eμ is required for V–D–J joining and for early IgH gene expression; 3′αE becomes active late in B cell development and participates in the Ig heavy chain class switch.

In the present study we have induced deletion of 3′αE in a cell line (9921) that already lacks Eμ. We undertook these experiments to determine whether 3′αE was responsible for sustaining IgH gene expression in Ig-secreting cells that lacked Eμ. As will be discussed below, 3′αE is not the only enhancer that has been identified 3′ of the IgH locus (Giannini *et al.*, 1993; Matthias and Baltimore, 1993; Madisen and Groudine, 1994). However, it was the first to be identified and, therefore, the first candidate considered in our experiments. Deletion of this element *in situ* allowed us to assess 3′αE function within its normal chromosomal context. 3′αE lies 70 kb downstream of the 9921 γ2a gene (Lieberson *et al.*, 1991). The same spacing between enhancer and promoter would be difficult to achieve in conventional reporter gene constructs. Moreover, it is not presently understood whether the nature of

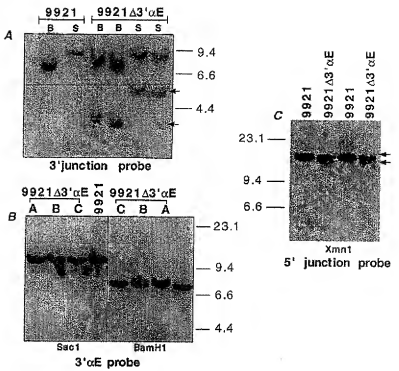

Fig. 2. Southern analyses demonstrating targeted deletion of the 3′αE region in 9921. Genomic DNA was isolated from the parental line (9921) and from a G418^r derivative line (9921Δ3′αE), digested with **(A** and **B)** *Bam*HI (B) or *Sac*I (S) or **(C)** *Xmn*I and size-fractionated on agarose gels. Nylon blots of the DNA were then hybridized to a 3′ junction probe (A), 3′αE sequences (B) or to a 5′ junction probe (C). The 5′ and 3′ junction probes are designated probes a and b respectively in the maps of Figure 1. The 3′αE probe is designated c in Figure 1. In (A) the new fragments generated upon homologous recombination of RVA with genomic sequences are indicated by arrows. In **(B)** DNAs from three subclones of the 9921Δ3′αE line are given letter designations (A, B and C). The two arrows in (C) mark the closely migrating *Xmn*I fragments derived from IgH chromosomes that retain the 3′αE region (upper fragment) and the IgH chromosome that has undergone replacement of the 3′αE region with *neo* sequences (lower fragment) respectively. Molecular size markers indicated (23.1, 9.4, 6.6 and 4.4 kb) are *Hind*III fragments of bacteriophage λ.

the DNA separating an assembled IgH gene and a distal enhancer, such as 3′αE, might affect their interactions with one another (e.g. the presence of matrix attachment sites; Cockerill, 1990).

Our studies show that 3′αE is required for γ2a gene transcription in the 9921 cell line. Targeted deletion of a 5.1 kb region that included 3′αE gave rise to a cell line that produced normal levels of Ig κ light chain, but which had ceased production of γ2a heavy chain. This DNA region, therefore, strongly regulates the activity of a promoter over 70 kb away. As discussed below, the finding that 3′αE behaves as a strong transcriptional enhancer *in situ* and in the absence of Eμ is also of relevance to the deregulated expression of translocated c-*myc* genes in tumors of the B cell lineage.

Results

Isolation of a 9921 subclone that has undergone deletion of 3′αE sequences

In order to induce deletion of 3′αE from the active IgH locus of 9921, we constructed the targeting vector replacement vector A (RVA; Figure 1). This construct was designed so that recombination between it and the targeted chromosome would result in replacement of 5.1 kb of genomic DNA with 1.9 kb of exogenous DNA encompassing a selectable marker gene (neomycin resistance gene, *neo*^{r}). The genomic DNA to be deleted included 3′αE and two overlapping DNase I hypersensitive sites identified by others (HS1 and HS2; Giannini *et al.*, 1993; Madisen and Groudine, 1994).

The regions of homology between the RVA targeting vector and genomic sequences consisted of 7 kb of 5′ flanking sequence and 1.35 kb of 3′ flanking sequence (see Figure 1). The *neo*^{r} gene lying between these flanking sequences was under the control of the phosphoglycerate kinase gene (*pgk-1*) promoter region. The RVA vector was linearized and introduced into 9921 cells by electroporation. A genomic DNA screen was used to identify geneticin-resistant (G418^r) transformants that had undergone the desired homologous recombination event. As diagrammed in Figure 1, homologous recombination between RVA and genomic DNA should result in novel *Bam*HI (3.7 kb) and *Sac*I (5.9 kb) fragments that would hybridize with a '3′ junction' probe (probe b in Figure 1). DNA from one of 450 screened clones generated the expected *Bam*HI fragment and was further analyzed with the *Sac*I enzyme (Figure 2A). A *Sac*I fragment of the expected size (5.9 kb) was also detected in this clone, so that it was provisionally designated 9921Δ3′αE. As would be predicted, neither the novel *Sac*I fragment nor the novel *Bam*HI fragment hybridized to a 3′αE probe (Figure 2B).

Southern analyses of 9921Δ3′αE revealed not only the novel *Bam*HI and *Sac*I fragments associated with the homologous recombination event, but also the *Bam*HI and *Sac*I fragments characteristic of non-transfected 9921 cells

(Figure 2A). This was an expected finding because of the chromosomal constitution of the 9921 cell line. One copy of the IgH chromosome (chromosome 12) in 9921 carries the functionally rearranged and expressed γ2a heavy chain gene. The other copy of chromosome 12, harboring the 'excluded' (non-expressed) IgH locus, has undergone reciprocal translocation with c-*myc* on chromosome 15 (see maps, Figure 4A; Stanton *et al.*, 1984). Subsequent to this translocation event, the reciprocal translocation products have undergone reduplication and, in previous studies, we have estimated that both are present at two or three copies per cell (Stanton *et al.*, 1984). One of the translocation products (designated M11myc3' in Stanton *et al.*, 1984) retains IgH locus sequences homologous to the RVA vector ('Translocated' chromosome in Figure 4A). As a result, RVA could recombine with either this translocated chromosome or with the copy of chromosome 12 carrying the functional γ2a gene ('Nontranslocated' in Figure 4A). Regardless of which chromosome underwent recombination with RVA, two or more additional chromosomes would retain the 3'αE region in its original configuration. As a result, the pattern of hybridization to the 3' junction probe should include both the original fragments detected in 9921 and the novel fragments resulting from RVA/genomic DNA recombination. Certainly, only when RVA recombined with the non-translocated chromosome carrying the functional γ2a gene would it be expected to affect IgH gene expression. This issue is considered in detail below. However, we first extended our analysis of the recombination event in 9921Δ3'αE to confirm that the expected replacement of sequences had taken place.

Having determined that the 3' flanking sequences of RVA had homologously recombined with IgH chromosomal sequences, we next looked for evidence that a second cross-over event had taken place within the RVA 5' flanking sequences. As diagrammed in Figure 1, the expected recombination event would generate a novel *Xmn*I fragment that could be detected with a '5' junction' probe (probe a, Figure 1). The new *Xmn*I fragment should be slightly smaller than that which would be detected with the 'a' probe before recombination (compare 'genomic' and 'recombinant' maps, Figure 1). When 9921Δ3'αE DNA was digested with *Xmn*I, the expected pattern of two closely migrating fragments was seen, confirming that this cell line carried an IgH chromosome that had undergone the desired homologous recombination event (Figure 2C).

The original 9921Δ3'αE isolate was subcloned by limiting dilution to ensure that it was a homogeneous population of cells. Genomic DNA analyses of each of the subclones showed the same patterns as those of the original cell isolate. Interestingly, hybridizations with a *neo*^{r} probe revealed that additional copies of the RVA vector had been integrated at random sites within the 9921Δ3'αE genome (data not shown).

9921Δ3'αE and subclones no longer produce γ2a mRNA or protein

To determine whether the loss of 3'αE sequences in 9921Δ3'αE had affected γ2a expression, we prepared cytoplasmic lysates and culture supernatants from five 9921Δ3'αE subclones. These were analyzed by enzyme-linked immunoassay (ELISA) for γ2a protein (Figure 3A). While lysates and supernatants from the parental 9921 line and from the MPC11 myeloma contained abundant γ2a and γ2b heavy chain protein respectively, no Ig heavy chain was detected in any of the 9921Δ3'αE subclones (data for 9921 and a representative 9921Δ3'αE subclone, Figure 3A). Furthermore, there was no γ2a mRNA in RNA samples prepared from three representative 9921Δ3'αE subclones (Northern blot analyses, Figure 3B). γ2a expression was specifically affected in these cell lines. Quantitative ELISAs demonstrated that all clones continued to produce κ light chain protein at the same levels as 9921 (data not shown) and κ mRNA was readily detectable by Northern analyses (Figure 3B).

It has been shown previously that myelomas give rise to Ig non-producing cell lines at a relatively high rate ($1/10^3$/cell/generation; Coffino and Scharff, 1977). While this rate is well above that for other loci in these or other cell types (generally $1/10^6$/cell/generation), it is low enough to suggest that we would not recover cells that had spontaneously lost Ig expression in a random screen of 450 myeloma subclones. Consistent with this prediction, we found that in a screen of 325 random transformants of the 9921 cell line, none had ceased to express Ig. Each of the transformants included in this screen had been transfected with plasmid DNA and isolated by growth in selective growth medium. However, none of them had undergone homologous recombination with the RVA vector (data not shown).

In almost all cases in which spontaneous Ig non-producing clones have arisen in cultures of myeloma cell lines (18 in a study of 19 spontaneous myeloma variants), the loss of IgH expression has resulted from detectable alterations in DNA: either loss of the relevant IgH chromosome or DNA rearrangements within or around the Ig heavy chain transcription unit (Yu and Eckhardt, 1986). We analyzed 9921Δ3'αE DNA, therefore, for evidence of either of these events. 9921Δ3'αE and parental 9921 DNA were both digested with *Bam*HI and *Sac*I and hybridized with a DNA probe (pJ11) that detects sequences within the γ2a transcription unit of 9921 (see map, Figure 4A). 9921 and the 9921Δ3'αE subclones were indistinguishable in these analyses, demonstrating that the 9921Δ3'αE subclones retained the γ2a gene actively transcribed in 9921 (Figure 4B). The lack of transcription at this locus in 9921Δ3'αE and subclones, therefore, is not likely to be due to spontaneous mutational events, but rather results from the homologous recombination event that has deleted 3'αE-region sequences ~70 kb downstream.

RVA-induced deletion of 3'αE has occurred in cis to the γ2a gene expressed in 9921

As noted earlier, the expressed γ2a gene of 9921 lies on an intact copy of chromosome 12 with 3'αE lying ~70 kb downstream (with respect to IgH gene transcription). The homologous IgH chromosome has undergone reciprocal translocation with chromosome 15 such that one of the translocation products (M11myc3') retains 3'αE and flanking sequences. The translocation has resulted from a break within the tandemly repetitive sequences 5' of Cγ2a (Sγ2a) on the IgH chromosome and a break within exon 1 of c-*myc* on chromosome 15 (Stanton *et al.*, 1984). The M11myc3' translocation product carries 3'αE *in cis* to the disrupted c-*myc* gene (~50 kb upstream with respect to

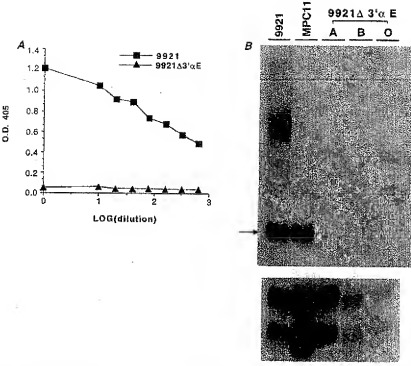

Fig. 3. γ2a heavy chain expression has ceased in 9921Δ3′αE. **(A)** Quantitative ELISA comparing γ2a protein levels in 9921 and 9921Δ3′αE. Titration curves of cytoplasmic lysates from 9921 and from a representative 9921Δ3′αE subclone are shown. Culture supernatants and cytoplasmic lysates from four additional 9921Δ3′αE subclones were also tested by quantitative ELISA for the presence of γ2a protein and similar results were obtained. γ2a heavy chain was detected with an alkaline phosphatase-conjugated rabbit antibody (**Materials** and methods). The reaction of this reagent with *p*-nitrophenol phosphate yields a colored product with an absorbance peak at 405 nm. **(B)** Northern blot analysis of total RNA isolated from 9921, the myeloma cell line MPC11 (γ2b, κ producer) and three representative 9921Δ3′αE subcloned lines (designated A, B and O). Aliquots of 20 μg total cellular RNA were included in each sample. Positions of ribosomal RNAs (28S and 18S) are indicated. The probe used in the upper blot was a γ2a cDNA probe (Zaller and Eckhardt, 1985) which also cross-hybridizes to γ2b transcripts. An arrow points to the γ2a and γ2b mRNA present in 9921 and MPC11 respectively. Subsequent hybridization of the same Northern blot with a probe specific for κ light chain mRNA, Cκ (Radomska *et al.*, 1994), revealed the two κ light chain mRNAs characteristic of the MPC11 lineage (including 9921). The faster migrating band is a truncated κ mRNA from the non-productive Ig κ allele; the more slowly migrating mRNA encodes functional κ light chain (Schibler *et al.*, 1978).

c-*myc* transcription; see map, Figure 4A). Our Southern blot data for 9921Δ3′αE indicated that one copy of 3′αE and its flanking sequences had been replaced by *neo*^{r} but that other copies of 3′αE remained intact in this cell line. Although the loss of γ2a expression in 9921Δ3′αE and its subclones strongly implied that the deletion had occurred *in cis* to the γ2a heavy chain gene on the non-translocated IgH chromosome, we undertook experiments to physically map the deletion to this or the translocated chromosome, M11myc3′.

M11myc3′ and the γ2a gene-containing IgH chromosomes are nearly identical throughout at least a 50 kb region surrounding 3′αE. This made it difficult to link *neo*^{r} sequences to one or the other chromosome by conventional genomic Southern analysis. Instead, we elected to use the method of somatic cell fusion as a means to physically separate the two chromosomes so that we could unambiguously map the homologously recombined copy of RVA to one or the other of these chromosomes. 9921Δ3′αE cells (G418^r) were fused to ouabain-resistant, Chinese hamster ovary (CHO, Ouabain^r) cells with the expectation that the resulting hybrids would have lost murine chromosomes during the process of cell fusion (Ruddle, 1972). An electrofusion method developed in our laboratory (Radomska *et al.*, 1994; Radomska and Eckhardt, 1996) was used for these experiments and hybrids were isolated after growth in medium containing ouabain and G418 (see Materials and methods). The use of G418 allowed us to select for hybrids that retained the chromosome in which 3′αE had been replaced by *neo*^{r}.

DNA samples from individual hybrid lines were subjected to Southern blot analysis using the *Bam*HI restriction enzyme and the H3.1 probe indicated in Figure 4A. The H3.1 probe hybridizes to two *Bam*HI fragments (6.7 and 5.8 kb) that map within the non-translocated (γ2a gene-containing) IgH chromosome and to a 5.4 kb *Bam*HI fragment from M11myc3′. The latter *Bam*HI fragment spans the translocation break point within the c-*myc* gene. It is possible to determine which hybrid lines retain which of these two IgH chromosomes (translocated or non-translocated) by determining which *Bam*HI fragments remain (i.e. all three, 6.7 and 5.8 kb only or 5.4 kb only).

Representative data from these analyses are shown in Figure 4C. All of the tested CHO×9921Δ3′αE hybrids (14/14) retained the 6.7 and 5.8 kb *Bam*HI fragments, demonstrating that all retained the intact copy of chromosome 12 carrying the expressed γ2a gene. However, most of the hybrids (12/14) had lost all copies of M11myc3′, as indicated by the absence of the 5.4 kb *Bam*HI fragment in these DNA samples. Preferential retention of the chromosome carrying the γ2a gene in the hybrid lines indirectly suggested that RVA (with *neo*^{r}) had integrated

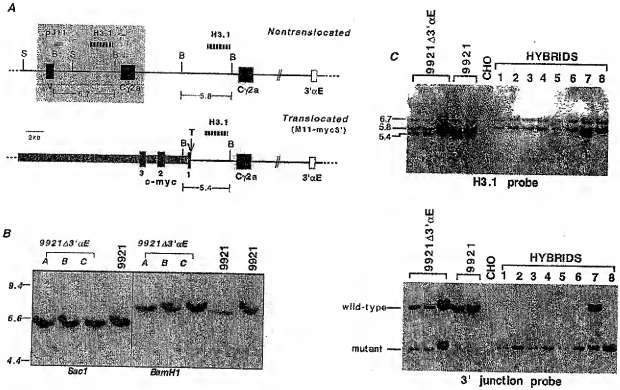

Fig. 4. Deletion of the 3′αE region has occurred *in cis* to the γ2a IgH transcription unit. **(A)** Two schematics of 9921 chromosomes are shown. One is of the IgH chromosome that carries the functional γ2a gene of 9921 ('Nontranslocated'). The γ2a transcription unit on this chromosome is highlighted. The allelic IgH chromosome in 9921 has undergone a reciprocal translocation with chromosome 15. The translocation break points map within c-*myc* (on chromosome 15) and upstream of Cγ2a (on chromosome 12) (Stanton *et al.*, 1984). The second schematic is of one of the products of this reciprocal translocation ['Translocated' (M11-myc3′); Stanton *et al.*, 1984]. T marks the site of recombination between chromosomes 15 and 12. The thick shaded line signifies sequences derived from chromosome 15. V_H, Cγ2a and c-*myc* exons are shown as black boxes. The *Bam*HI (**B**) and *Sac*I (S) restriction enzyme sites germane to the Southern analysis shown in **(B)** and **(C)** are indicated. Sequence homology to the pJ11 and H3.1 DNA probes is indicated above each map. The sizes of *Bam*HI fragments detected with these two probes are indicated below the maps (in kb). It should be noted that a second copy of Cγ2a lies 3′ of the γ2a transcription unit in 9921. This is the result of a tandem duplication which originally took place in MPC11, the cell line from which 9921 was derived (Tilley and Birshtein, 1985). Due to this duplication the H3.1 probe detects two distinct *Bam*HI fragments that come from the non-translocated (γ2a-producing) IgH chromosome. **(B)** Southern analyses of 9921 and 9921Δ3′αE subclones using a DNA probe specific for the γ2a transcription unit. DNAs from the cell lines were digested with *Sac*I (left) or *Bam*HI (right) and hybridized to pJ11 (see map in A). Molecular weight markers are as in Figure 2. **(C)** Southern analyses of CHO×9921Δ3′αE hybrids. *Bam*HI-digested DNA was prepared from 9921Δ3′αE, 9921, CHO and several individual hybrid lines (hybrids 1–8 are shown). Duplicate blots were prepared and one of these hybridized with the H3.1 probe. The *Bam*HI fragments detected with this probe are indicated (5.4,5.8 and 6.7 kb) and correspond to the fragments diagrammed in (A). A duplicate blot was hybridized with the 3′ junction probe (probe b in Figure 1). The two *Bam*HI fragments detected with this probe come from a region 3′ of Cα and signify either RVA-induced disruption of this region ('mutant' fragment) or no disruption of 3′αE flanking sequences ('wild-type' fragment).

into that chromosome. This was confirmed by additional Southern blot analyses.

Duplicate samples of *Bam*HI-digested genomic DNA were hybridized with the 3′ junction probe (probe b in Figure 1) to determine which hybrids carried the mouse chromosome that had homologously recombined with RVA. Since analyses with the H3.1 probe showed that all hybrids retained the non-translocated IgH chromosome (with the assembled γ2a gene), the prediction was that all hybrids would also retain the diagnostic *Bam*HI fragment associated with RVA-induced deletion of 3′αE, if this deletion had occurred *in cis* to the γ2a gene. Consistent with that prediction, all 14 hybrids analyzed retained the novel 3.7 kb *Bam*HI fragment derived from the RVA-targeted allele (Figure 4C; compare with Figure 2A). Notably many of these hybrids lacked M11myc3′, proving that RVA had not homologously recombined with this translocated chromosome. Rather, the 6.9 kb *Bam*HI fragment characteristic of an undisrupted 3′αE region was mapped to the M11myc3′ chromosome, since this *Bam*HI fragment was present only in those two hybrids that also retained a copy of M11myc3′ (data are shown for only one of these two hybrids, Figure 4C lane 7). Taken together, these data clearly show that RVA-induced deletion of the 3′αE region has occurred *in cis* to the γ2a transcription unit, which has simultaneously ceased to function.

Discussion

Over 10 years ago we and others described a number of Ig-producing cell lines that expressed Ig heavy chain in the absence of Eμ (Klein *et al.*, 1984; Wabl and Burrows, 1984; Aguilera *et al.*, 1985; Zaller and Eckhardt, 1985). At the time this was the only known transcriptional enhancer within the IgH locus. The discovery that IgH genes could function without Eμ provided the earliest

indication that there were additional elements participating in the control of IgH gene expression.

In recent years several additional enhancers have been mapped to the IgH locus, but their respective roles in promoting and/or regulating endogenous IgH gene expression remain unclear. To explore further the function of one of these enhancers, 3′αE, we have made use of one of the Eμ-independent cell lines, 9921. 9921 expresses γ2a heavy chain from an IgH allele that lacks Eμ. In fact, Eμ is altogether missing from the genome of this cell line (Eckhardt and Birshtein, 1985). 3′αE, on the other hand, is on both the IgH allele that produces γ2a transcripts and on a homologous allele, where it lies 50 kb upstream of a translocated *c-myc* locus. In this study we have shown that when the 3′αE region *in cis* to the γ2a gene is replaced with foreign DNA sequences the γ2a gene ceases to function. This finding strongly supports the notion that this distant 3′ region serves an essential function in the transcription of assembled IgH genes.

There have been a number of experiments involving mice in recent years which also bear on the question of enhancer function within the IgH locus. Chimeric mice have been produced in which B lymphocytes lacked Eμ on one of the IgH alleles. These mice showed greatly impaired V–D–J joining on the Eμ-deficient IgH chromosome, suggesting both that efficient V region gene assembly requires Eμ and that enhancer regions 3′ of the IgH locus cannot supplant Eμ in this process (Chen *et al.*, 1993; Serwe and Sablitzky, 1993). In other recent experiments in mice, 3′αE and surrounding sequences (5.5 kb deletion) were deleted in the lymphoid progenitors of chimeric mice (Cogne *et al.*, 1994). In these mice there was no apparent effect on V–D–J joining, but Ig heavy chain class switching was impaired. The emerging picture is one in which Eμ plays important roles in the activation and regulation of IgH genes in early B cells, while 3′ IgH region sequences take on greater importance as B cells mature and/or are activated by antigen.

A limitation to studies involving gene manipulations in mice is that a block in cell development, due to loss of a necessary gene or control region, precludes analysis of the same gene's or control region's function at later developmental stages of the same cell lineage. However, this limitation can be overcome by studies of appropriate cell lines. For example, spontaneous deletion of Eμ within an assembled heavy chain gene of several pre-B cell lines resulted in loss of IgH gene expression, demonstrating that Eμ not only serves to promote efficient V–D–J assembly (as suggested by the mouse studies), but is also required for transcription of IgH genes soon after they are formed (Alt *et al.*, 1982; Wabl and Burrows, 1984). Interestingly, the Eμ-deficient IgH gene of one of these pre-B cell lines could be reactivated under conditions that would also activate 3′αE (Alt *et al.*, 1982; Wabl and Burrows, 1984; Arulampalam *et al.*, 1994). The 3′αE deletion studies in mice suggest that the 3′ IgH region, like Eμ, plays an important role in DNA recombination (in this case heavy chain class switch recombination; Cogne *et al.*, 1994). However, the results presented here reveal an additional, important function for 3′αE as a transcriptional enhancer for the assembled IgH genes expressed in Ig-secreting cells.

It is important to point out that in the experiments we describe here and in the 3′αE deletion in mice (Cogne *et al.*, 1994), a selectable marker was inserted in place of deleted genomic DNA sequences. This was also true for one of the Eμ deletion studies in mice (Chen *et al.*, 1993). In all of these cases it is possible that substitution of the respective enhancers with a marker gene (in our case *neo*^{r}) does not constitute a true 'null' mutation. It has been reported that insertion of marker genes within the locus control region (LCR) of the β-like globin locus can, by itself, negatively affect expression of the β-like globin genes (Kim *et al.*, 1992; Fiering *et al.*, 1993). These results were interpreted as evidence that the LCR elements could be redirected to serve a more proximal promoter (that of the reporter gene) when the latter was provided by gene insertion. Certainly, this finding raises the possibility that the effects we have seen in 9921 and that were seen in mice upon 3′αE deletion were due to diversion of control elements that map outside the deletion itself, away from the IgH promoter. This possibility can be directly tested by modification of the RVA replacement vector to permit subsequent deletion of *neo*^{r}, and such studies are underway. In any case, there are examples in which insertion of reporter genes within the IgH locus had little or no effect on Ig gene activity (Baker *et al.*, 1988; Chen *et al.*, 1993). The surprising effect of reporter gene insertion in the β-like globin locus LCR may be related to the fact that stage-specific control of the β-like globin genes relies directly upon competitive interactions between promoters and enhancers.

While our studies demonstrate that disruption of the 3′αE region silences the γ2a gene in 9921 cells, it should not be assumed, on the basis of this finding, that 3′αE is sufficient to sustain expression of this gene. In our earlier studies of 3′αE we found it to be a less potent enhancer than Eμ in transient assays in the J558L myeloma cell line (Lieberson *et al.*, 1991). Stable transfection assays in 9921 using a reporter gene under the control of Eμ or of a 4 kb *Xba*I fragment containing 3′αE also suggest that 3′αE is a less efficient transcriptional enhancer (R.Lieberson and L.A.Eckhardt, unpublished results). However, several additional regulatory elements have recently been described in the 3′ flanking region of the IgH locus, any one or all of which could work together with 3′αE to achieve high level IgH gene transcription in Ig-secreting cells. A region with relatively weak transcriptional enhancement activity has been identified immediately 3′ of Cα membrane sequences (Cα3′E; Matthias and Baltimore, 1993). In addition to the DNase I hypersensitive sites that map within and adjacent to 3′αE (HS1 and HS2), there are two DNase I hypersensitive sites that map 13 (HS3) and 17 kb (HS4) farther downstream (Giannini *et al.*, 1993; Madisen and Groudine, 1994). HS3 was identified in a number of myeloma cell lines, but was absent in DNA preparations from both non-B cells and earlier stage B cells (Madisen and Groudine, 1994). HS4 was identified as a B cell-specific site present in both pre-B and plasmacytoma lines and acts as an enhancer element in transfection assays (Giannini *et al.*, 1993; Madisen and Groudine, 1994; Michaelson *et al.*, 1995). The combination of four DNase I hypersensitive sites (HS1, HS2, HS3 and HS4) has recently been described as an LCR, due to its ability to promote copy number-dependent, insertion site-independent expression of

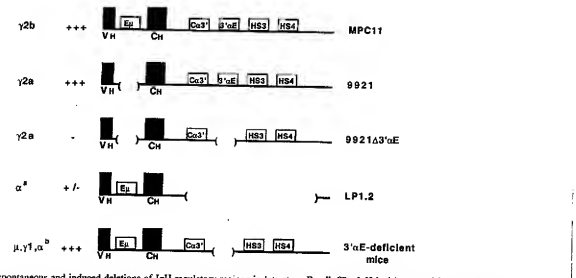

Fig. 5. Schematic of spontaneous and induced deletions of IgH regulatory regions in late stage B cells.The IgH loci in several Ig-producing cells are diagrammed (not to scale). Heavy chain variable (V_H) and constant (C_H) region coding sequences are represented as filled boxes. The relative positions of the five control elements discussed in the text (Eμ, Cα3′, 3′αE, HS3 and HS4) are shown as open boxes. The names of the Ig-secreting cell lines are shown to the right of each map and the classes of heavy chain they produce are indicated to the left. Levels of heavy chain mRNA and protein in the cell lines are represented as +++ (for high level expression), +/- (low expression) and - (no expression). [a]The deletion variant LP1.2 produces α heavy chain at 10% of the level of the original parental cell line (Gregor and Morrison, 1986). [b]3′αE-deficient spleen cells from chimeric mice, when stimulated with LPS and lymphokines, secreted IgM, IgG_1 and IgA at levels comparable with those produced by wild-type spleen cells (Cogne *et al.*, 1994).

reporter genes in myeloma cells (Madisen and Groudine, 1994). If, as suggested by the latter studies, 3′αE is only one component of a larger functioning unit (LCR), then deletion of other single elements might yield the same phenotype we describe for 3′αE (loss of IgH gene expression). This is an issue that can be addressed in future deletion experiments in Ig-secreting cell lines.

While 3′αE is essential for IgH expression when Eμ is absent, it would appear that the other 3′ region elements (e.g. Cα3′E, HS3 and HS4) can function without 3′αE when Eμ is present. In mice carrying the 3′αE deletion (replacement) but with Eμ intact, spleen cells expressing some heavy chain isotypes (e.g. IgM, IgG1 and IgA) were reported to do so at normal levels, suggesting that 3′αE was not required for expression of these genes (see Figure 5). However, it cannot be concluded that Eμ alone was sustaining IgH expression in these cells. Several years ago a spontaneous deletion of sequences 3′ of Cα was described in an IgA-secreting cell line (LP1.2; Gregor and Morrison, 1986). Although Eμ remained within the α heavy chain gene of this cell line, the deletion of 3′ region sequences resulted in a dramatic reduction in IgH expression levels (to 10% of that of the parental line; Gregor and Morrison, 1986). The DNA deletion in LP1.2 involved ~40 kb, began just upstream of the Cα membrane exon and included all of the putative control elements that have been subsequently mapped 3′ of Cα (Figure 5). Without any of these 3′ region sequences it appeared that Eμ was insufficient to maintain the high levels of IgH expression characteristic of Ig-secreting cells. Similarly, Eμ may not act alone to achieve high level expression of IgH genes in the 3′αE deletion mice, but rather it may act in synergy with the remaining 3′ region control sequences (Figure 5). In summary, the regulation of IgH gene assembly, rearrangement and transcription in developing and activated B cells appears to require the action of multiple control elements. Further experiments are required to determine the precise nature of the contributions made by each of these elements to these several processes.

There is reason, separate from their relevance to IgH gene expression, to pursue experiments that allow analysis of 3′ IgH region function in the absence of Eμ. As noted in the Introduction, c-*myc* translocations with the IgH locus in mouse myeloma and human Burkitt's lymphoma frequently juxtapose c-*myc* not with Eμ, but with the much farther 3′ region of the IgH locus (reviewed in Cory, 1986). In this case oncogenic transformation of Ig-expressing cells results, in part, from deregulation of the c-*myc* gene upon placing it under 3′ IgH region control. It will be interesting to determine whether deletion of 3′αE and the HS1 and HS2 sites has as dramatic an effect on translocated c-*myc* expression in 9921 as it has on IgH expression.

Materials and methods

Cell lines

9921 is a γ2a/κ-producing Ig class switch variant which arose spontaneously from the cell line 971. 971 was, in turn, isolated from mutagenized 45.6.2.4 cells. This lineage is described in Eckhardt and Birshtein (1985). 45.6.24 is a tissue culture-adapted subline of the γ2b/κ-producing BALB/c mouse tumor MPC11 (Laskov and Schaff, 1970). In the present study we refer to this cell line as MPC11. OY21 is a ouabain-resistant variant of the Chinese hamster cell line CHO (a gift of L.Chasin; Urlaub and Chasin, 1980). 9921 was maintained in Dulbecco's modified Eagle's medium (DME; Gibco-BRL, Grand Islands, NY) with 15% horse serum (HyClone Laboratories, Logan, UT). OY21 was maintained in RPMI 1640 (Gibco-BRL, Grand Islands, NY) with 10% horse serum. All media contained 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. Cells were maintained at 37°C in an atmosphere of 7% CO_2.

Plasmid constructs

RVA was designed to induce a recombination event in which a 1.9 kb neomycin gene (*neo*[r]) would replace 5.1 kb of DNA encompassing 3′αE

(Lieberson *et al.*, 1991) and DNase I hypersensitive sites 1 and 2 (HS1 and HS2; Madisen and Groudine, 1994). RVA was constructed such that the *neo*^{r} gene lay between cloned genomic DNA sequences that mapped immediately upstream (5' flanking sequence) and downstream (3' flanking sequence) of the desired genomic DNA deletion. The 5' flanking sequence consisted of a 7 kb genomic DNA fragment isolated from the phage clone pλM2 (a gift of S.Pettersson and M.Neuberger; Dariavach *et al.*, 1991). This fragment was subcloned into the *Eco*RI and *Hin*dIII sites in the multiple cloning site region of pBS SK$^+$ (Catalog No. 21120, Stratagene, La Jolla, CA). The resulting plasmid was designated pRV5'. The 3' flanking sequence was a 1.35 kb *Apa*LI–*Eco*RI fragment which was also subcloned from the pλM2 phage. This fragment was introduced into the *Hin*dIII site of the pIC19R/MC1-TK plasmid by blunt-end ligation (pIC19R/MC1-TK was kindly provided by M.Cappechi; Mansour *et al.*, 1988). The resulting plasmid (pRVA-3'tk) carried both the desired 3' flanking region and the negative selection marker, HSV-tk (McKnight, 1980). A 3.5 kb *Xho*I–*Sal*I fragment from pRVA-3'tk was then inserted into a *Sal*I site in pRV5'. The resulting plasmid, RVAΔneo, consisted of both the 5' and 3' flanking sequences and the negative selection marker, HSV-tk. Finally, a 1.9 kb *Eco*RI–*Hin*dIII fragment that carried a truncated *neo*^{r} gene was inserted, by blunt-end ligation, into the single *Sal*I site of RVAΔneo. The *neo*^{r} gene was under the control of the phosphoglycerate kinase-1 (*pgk*-1) promoter and upstream activating element and was isolated from pKJ-1 (provided by F.Alt, Chen *et al.*, 1993). This resulted in the final construct, RVA, which is diagrammed in Figure 1.

Our initial plan was to use the positive–negative selection scheme devised by Mansour *et al.* (1988) so that we could select against clones that had integrated RVA at random locations within the genome. This selection scheme exploits the fact that DNA replication can be blocked in cells that produce herpes simplex virus thymidine kinase (HSV-tk) through the use of HSV-tk substrate analogs (e.g. gancyclovir and FIAU). As shown in Figure 1, the HSV-tk gene was placed at one end of the RVA recombination vector so that it would likely be retained if the vector integrated randomly within the genome. In preliminary experiments, however, we found that random integration of the HSV-tk gene in 9921 did not render these cells sensitive to either gancyclovir or FIAU. As a result we abandoned this strategy of enriching for homologous recombination events. Others have reported similar difficulty in achieving efficient killing of HSV-tk-expressing cells (e.g. Mombaerts *et al.*, 1991).

Cell transfections

DNA was introduced into 9921 cells by electroporation (Potter *et al.*, 1984; Potter, 1988). Aliquots of 20 μg of *Xho*I-linearized RVA plasmid DNA were combined with a 1 ml suspension of 10^7 9921 cells and the mixture dispensed into a 0.4 cm (width) electroporation cuvette (BTX, San Diego, CA). An electric pulse was delivered at 960 μF and 250 V by a Bio-Rad Gene Pulser™ electroporator and Capacitance Extender™ (Bio-Rad, Hercules, CA). The cells were then diluted in non-selective medium and plated at 10^5 cells/well in 96-well culture plates. After 72 h medium supplemented with 1.5 mg/ml G418 (Gibco-BRL, Grand Island, NY) was added to select for stable transformants expressing the *neo*^{r} gene. Colonies were visible 8–16 days after transfection. In most experiments transformants arose in 50–80% of the individual wells on each culture plate.

Cell fusions

OY21 cells (5×10^6) were fused with equal numbers of 9921Δ3'αE by an electrofusion technique developed in our laboratory (Radomska and Eckhardt, 1996). The two cell types were combined, washed twice in serum-free medium (RPMI) and then resuspended in 0.1 ml of the same serum-free medium. The cell suspension was dispensed into a 0.2 cm electroporation cuvette (BTX, San Diego, CA) and centrifuged at low speed to promote cell–cell contact. Cells then received a 250 V, 960 μF pulse delivered by a Bio-Rad Gene Pulser™ electroporator and Capacitance Extender™. After a 60 min incubation at 37°C in a 7.5% CO_2/air atmosphere the cells were resuspended in 25 ml RPMI 1640 medium containing 10% horse serum and transferred to a 150×25 mm tissue culture dish (Catalog No. 08-772-6, Fisher, Pittsburgh, PA). Forty eight hours post-fusion RPMI 1640 medium supplemented with 1×10^{-3} M ouabain and 1.25 mg/ml G418 was added to the fusion plate to select for hybrids. The OY21 line is resistant to ouabain, but sensitive to G418. The 9921Δ3'αE cell line is sensitive to ouabain, but carries the *neo*^{r} gene, making it resistant to G418. Fourteen G418-resistant, ouabain-resistant clones were selected for further analysis.

Southern blot analyses

Agarose gel electrophoresis, transfer to membrane and DNA hybridizations were performed essentially as described previously (Radomska *et al.*, 1994), with minor modifications. Briefly, ~18 μg restriction enzyme-digested DNA was loaded into each lane of a 0.7% agarose gel. Size-fractionated DNA was then transferred to Nytran (Schleicher & Schuell Inc., Keene, NH). Blots were pre-hybridized and hybridized at 65°C in buffer containing 7.5× Denhardt's solution, 3× SSC, 100 μg sonicated salmon sperm DNA and 0.5% SDS. Probes were labeled by the random primer method using a MegaPrime™ labeling kit (Catalog No. RPN1605, Amersham, Arlington Heights, IL). For all hybridizations the mouse sequences were separated from vector fragments before labeling and use as radioactive probes.

Probes included pJ11, 3'αE, H3.1, '5' junction fragment' and '3' junction fragment'. pJ11 is a *Bam*HI–*Eco*RI fragment derived from the J_H gene region of BALB/*c*J liver DNA and subcloned into pBR322 (Marcu *et al.*, 1980). 3'αE corresponds to the 596 bp enhancer element previously described by us (Lieberson *et al.*, 1991). H3.1 is a 2.4 kb fragment originally derived from the Ch 9.9.2.1 phage clone and corresponds to the *Hin*dIII fragment located directly 3' of the 9921 class switch site (Eckhardt and Birshtein, 1985). The 5' junction probe is a 2.8 kb *Sma*I–*Eco*RI fragment that contains the membrane exon of Cα. It was isolated from plasmid IgA3 (a gift of S.Morrison) which contains Cα sequences subcloned into pBR322 (Gregor and Morrison, 1986). The 3' junction probe is a 500 bp *Eco*RI–*Xba*I fragment derived from the 3' end of the 3.7 kb *Xba*I fragment which spans 3'αE (5'→3' position based on natural orientation with respect to IgH gene transcription).

Northern analyses

Total cellular RNA was isolated by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Approximately 20 μg total RNA/sample were analyzed. RNA samples were denatured with formamide, size-fractionated on 1% formaldehyde–agarose gels and transferred to Nytran essentially as described by others (Maniatis *et al.*, 1982). Blots were hybridized to ^{32}P-labeled DNA probes at 37°C for ~48 h in a buffer solution of 50% formamide, 7.5× Denhardt's solution, 5× SSC, 50 mM $NaPO_4$, pH 7.4, 100 μg/ml sonicated salmon sperm DNA and 0.5% SDS. To remove non-specifically bound probe, blots were washed once in 0.1× SSC, 1% SDS at 37°C for 30 min and an additional four times in the same buffer at 65°C (30 min per wash).

γ2a mRNA levels were assessed by using a cDNA probe that covers most of the constant region of γ2a (pγ2a-10-21; Auffray *et al.*, 1980). This probe detects both γ2a and γ2b transcripts. To verify the integrity of RNA samples and to determine relative mRNA levels among the samples, blots were stripped and rehybridized to a Cκ probe (Cκ; Radomska *et al.*, 1994) which detects Ig κ transcripts.

Enzyme-linked immunosorbent assays (ELISA)

Microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 10 μg/ml affinity-purified Fc fragment-specific rabbit anti-mouse IgG (Catalog No. 315-005-008, Jackson ImmunoResearch Labs, West Grove, PA). Coated wells were then incubated with 50 μl samples of cell culture supernatants or cell lysates. Cell culture supernatants were harvested from 48 h cultures of 1×10^6 cells and were serially diluted in the assay. Cell lysates were prepared by lysis of 1×10^6 cells in 50 μl 0.5% Nonidet P-40 lysis buffer (Zaller and Eckhardt, 1985). Again, serial dilutions of the lysates were assayed to determine Ig levels.

γ2a heavy chains were detected with alkaline phosphatase-conjugated rabbit anti-mouse γ2a (subclass specific; Catalog No. 315-005-008, Jackson ImmunoResearch Labs, West Grove, PA), using *p*-nitrophenol phosphate as the enzyme substrate (Catalog No. 104 phosphatase substrate tablets, Sigma, St Louis, MO). The absorbance at 405 nm was measured in an ELISA plate reader (Bio-Rad, Hercules, CA). For κ light chain-specific analyses plates were coated with 10 μg/ml affinity-purified goat anti-mouse κ antibody (Catalog No. OB1140-UNL, Fisher Biotech, Pittsburgh, PA). Bound κ chain was detected by incubation with biotinylated goat anti-mouse κ antibody (Catalog No. RPN1179, Amersham, Arlington Heights, IL) and subsequent addition of an alkaline phosphatase–avidin conjugate (Catalog No. 43-4422, ZYMED, South San Francisco, CA).

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Cutting Edge: Ig Heavy Chain 3′ HS1–4 Directs Correct Spatial Position-Independent Expression of a Linked Transgene to B Lineage Cells[1]

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The Ig H chain locus is regulated by a set of *cis*-acting elements. Hypersensitive sites (HS) located 3′ of the IgH, HS1–4, has been suggested to act as a locus control region (LCR) in cell lines. To assess the proposed role of HS1–4 acting as an LCR, we generated transgenic mice harboring a V_H promoter-β-globin reporter gene linked to the Ig H chain HS1–4 3′ regulatory sequences. Transgene expression is strictly confined to B lymphocytes, with no detectable expression outside the B cell lineage in all transgenic founder lines. Furthermore, reporter gene activity is integration independent but not copy number dependent. Thus, additional sequences are required to allow the HS1–4 regulatory region to act as a classical LCR in mice. Our data are discussed in the context of tissue-specific gene expression in B lineage cells. *The Journal of Immunology,* **1999, 163: 4637–4641.**

Regulation of transcription and rearrangement in the Ig H chain (IgH)[4] locus is tuned by a complex interplay of multiple regulatory elements. Germline transcription of the V_H and $C\mu$ region and initiation of VDJ rearrangements are regulated by upstream elements including the V_H promoter, the DQ52 promoter/enhancer, and the $E\mu$ enhancer (1). However, the problems to direct Ig-gene expression in a correct spatial and temporal fashion have focused the attention on additional regulatory elements located within the 3′end of the IgH locus (2). One of these enhancer elements, the IgH 3′ enhancer (3–6) (Fig. 1), has been shown to be active in late B cell development and can be activated in resting B cells in a ligand-receptor-dependent fashion (7, 8).

DNase I hypersensitivity assays and functional assays have revealed three additional transcriptional active enhancers (HS3a, HS3b, and HS4) in addition to the 3′enhancer (HS1,2) in the 3′end of the IgH locus (9–11) (Fig. 1). The overall structure of the IgH 3′ region encompassing the HS3a123b4 enhancer elements (referred to as HS1–4) consists of an over 20-kb long palindrome. Thus, HS3a and HS3b elements are part of large inverted repeats flanking the HS1,2 enhancer (12).

A number of Ig-transgenic lines, linked to V_H promoter-IgH-$E\mu$, have been generated with high levels of expression in B lymphocytes but always with aberrant expression outside B lineage cells. Similar data have also been observed with the HS1,2 enhancer-driven transgenes (7, 13).

A DNA fragment containing all 3′ HS sites except HS3a has previously been shown to direct position-independent and copy-dependent expression of a linked *c-myc* gene that was integrated as a stable transfectant in a plasmacytoma cell line. The authors suggested that the 3′end of the IgH locus might act as a locus control region (LCR) (11). An LCR, as originally identified in the β-globin locus, is defined functionally by its ability to direct tissue-restricted expression of a linked gene in a position-independent, but copy number-dependent, manner (14–17).

To assess the purported role of the IgH 3′ region, acting as an LCR, transgenic mice carrying all the HS1–4 3′ IgH enhancers were generated. Whereas this region directs correct B cell-specific expression of a linked V_H promoter-dependent reporter gene, no strict copy-dependent expression is observed. Thus, the mechanism of action of the 3′ regulatory region on the IgH locus, and IgH gene expression, is more complex than previously anticipated.

Materials and Methods

Construction of plasmid for microinjection

The pV_H-LCR vector contains all four 3′ IgH enhancers inserted downstream of the reporter gene. The HS1,2 is a 0.6-kb *StuI-EcoRV* DNA fragment (3, 4). HS3a and HS3b are duplicated enhancers flanking the HS1,2 enhancer but orientated in opposite directions on the chromosome. Two 2.1-kb *EcoRI-HindIII* genomic fragments, HS3a and HS3b, respectively, were prepared and inserted on both sides of the HS1,2 enhancer, thus mimicking the endogenous configuration (10, 18). HS4 is the 1.38-kb *PstI-HindIII* DNA fragment (11). The pV_H promoter is a 0.2-kb *HindIII* fragment derived from a rearranged murine V_H segment.

Generation of transgenic mice

A purified pV_H-LCR DNA fragment was used to generate transgenic founder lines (7). Positive founders were identified by PCR and Southern

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[4] Abbreviations used in this paper: IgH, Ig H chain; LCR, locus control region; HS, hypersensitive site; HSP, heat shock protein; RPA, ribonuclease protection assay.

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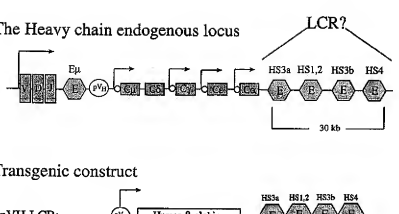

FIGURE 1. Schematic representation of the endogenous H chain locus and the pV_H-LCR construct used to generate the transgenic mice. Enhancers are denoted E, with their individual name indicated above; the H chain promoter is denoted pV_H. The constant region genes are indicated by filled boxes whereas the VDJ sequences are marked in gray. Shown is also the size of the 3′ regulatory region.

blot analysis. F_2 animals were analyzed for expression and used in all subsequent experiments. The sequences of the oligonucleotides used as primers in the PCR were: CAG GTG CAC CAT GGT GTC (including the *Nco*I site of the β-globin gene) and AAG CTT GAA AAC CTC AGA GGA (including the *Hin*dIII site of pV_H).

RNA extraction and ribonuclease protection assay

Total RNA from different organs was extracted and analyzed by ribonuclease protection assay (RPA) (3). The riboprobe, which spans the V_H promoter and 52 bp from the β-globin gene, was PCR amplified from the pV_H-LCR vector and cloned into a topo-cloning vector (Invitrogen, San Diego, CA). A 390-bp nonprotected radioactive riboprobe was achieved by in vitro transcription of an *Eco*RV cut vector with SP6 polymerase (Promega, Madison, WI) in the presence of [α-^{32}P]UTP (Amersham, Arlington Heights, IL). This probe generated a 160-bp protected fragment when hybridized to a correctly initiated transcript. The heat shock protein, HSP70, used as internal standard, as well as the $C\mu$ riboprobe, has been described previously (19).

Enrichment of B and T lymphocytes

Isolation of B and T lymphocyte populations was made from single cell suspensions of whole spleen. Splenic T cells were incubated with anti-Thy1.2-coated magnetic beads and isolated on a MiniMACS column according to instructions (Miltenyi Biotech, Auburn, CA). The B cell population were separated on a MiniMACS column following incubation with mouse anti-B220. The isolated cell populations were analyzed for enrichment on a FACScan.

RT-PCR analysis

First strand syntheses were performed on RNA from the different cell populations using the Ready To Go Kit (Pharmacia Biotech, Uppsala, Sweden) as described in the manual. The following primers were used for the PCR amplification: β-globin transgenic, upper, 5′-TGGTGGTCTACCCTTGG-3′; β-globin transgenic, lower, 5′-AAGAAAGCGAGCTTAGTGAT-3′; Btk, 5′-CTGGAGAGCATCTTTCTGAA-3′; Btk, 5′-CTTCTCGGAATC TGTCTTTC-3′; and GAPDH (Clontech, Palo Alto, CA). The PCR reactions were performed under the following conditions for 30 cycles: 1 min denaturation at 94°C, 1 min annealing at 54°C, and 2 min at 72°C.

Determination of copy number

Tail DNA (10 μg) was digested with *Eco*RI and probed with a 2.3-kb fragment, covering a large portion of the β-globin gene and the V_H promoter, generated from an *Eco*RI digest of the −128 3′ E plasmid (7). To determine the copy number of the individual founder animals, the intensity of the bands, quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA), was compared with an internal standard. To ensure that the amount of DNA was equivalent, the same blot was subsequently probed with a probe specific for HS4. The primers used to generate the HS4 probe were as follows: *Hin*dIII site, 5′-AGGTTGGGTTGGTCACCAGATTCT-3′; *Pst*I site, 5′-CTGCAGACTC ACTGTTCACCATG-3′.

Results

Generation of transgenic mice

To assess whether the enhancers in the 3′end of the IgH locus could act as an LCR, we generated transgenic mice harboring a natural V_H promoter-β-globin reporter gene potentiated by HS1–4 inserted 3′ of the reporter gene. The construct (pV_H-LCR) thereby mimics the endogenous locus in orientation and relative order (Fig. 1). Six independent founder lines, denoted I-VI, were established and analyzed in detail. Expression of the β-globin reporter gene was tested by RPA. The riboprobe generated a 160-bp protected fragment appearing as a double band (Fig. 2). The upper band represented the specific transcript whereas the lower represented a cryptic transcript. As shown in Fig. 2, all of the six founder lines were found to express the correct transcript of the reporter gene in RNA prepared from spleen, although at different levels.

B cell-specific expression of the transgene

To examine whether the transgene was expressed in a tissue-specific manner, RNA was prepared from different organs (spleen, heart, liver, kidney, brain, and thymus) and was determined by RPA. All six founders displayed tissue-specific expression of the transgene; high levels of transgene expression were observed in splenic cells. No expression was detected in the nonlymphoid tissues. Fig. 3 shows the RPA analysis of three of the founder lines. In addition, a weak signal was observed in thymus. To further

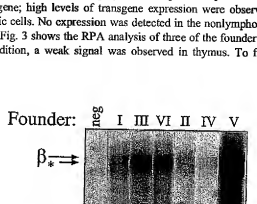

FIGURE 2. All founders express the correct transcript. RPA with splenic RNA showing the correct transcript (β) marked by an arrow and internal standard HSP70. Indicated above is the number of the founder line as well as a negative mouse line. The unspecific transcript is marked by an asterisk.

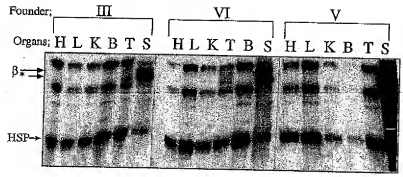

FIGURE 3. Expression of the transgene is tissue restricted. Transgenic lines were examined for expression in various organs. RPA showing the expression pattern of the transgene of the three representative transgenic lines. H, heart; L, liver; K, kidney; B, brain; T, thymus; and S, spleen. Arrows show the migration of the specific transcript (β), the cryptic transcript (*), and the internal standard (HSP).

examine the expression observed in thymus, we included a Cμ riboprobe, which detects endogenous Cμ gene expression in B lymphocytes, and hence indirectly the number of B cells (Fig. 4*A*). All the thymus RNAs examined contained relatively high levels of the Cμ transcript (Fig. 4*A*), and, since T cells express only marginal levels of Cμ (20), we concluded that the Cμ expression observed originates mainly from activated B cells contaminating the thymus preparations. In addition, RT-PCR analysis was performed, using MACS-sorted splenic B cells as well as T lymphocytes isolated from spleen and thymus originating from the high copy number founder V. The purity of the sorted cells was determined by FACS analysis and found to be 98% for B lymphocytes, 60% for the T cells isolated from spleen, and 99% for thymus (data not shown). To further select for a pure T cell population, Thy1-enriched splenic T cells were stimulated with anti-CD3 for 48 h in vitro. As a control for B cell contamination, we used the expression of the cytoplasmic tyrosine kinase Btk, known to be expressed in B cells but not in T cells (21). GAPDH was used as control for the amount of cDNA. As shown in Fig. 4, expression of the transgene was exclusively found in B lymphocytes or in T cell preparations containing contaminating B lymphocytes, as indicated by the positive signal from the Btk control. Moreover, no transgene expression was detected in the anti-CD3-activated pure T lymphocyte population or in the purified T cells from the thymus, where also no detectable amount of B cell contamination was observed, determined by the absence of a positive Btk signal (Fig. 4). In conclusion, these data show that the pV_H-driven transgene under the control of HS1–4 is expressed in a stringent tissue-specific manner in B cells.

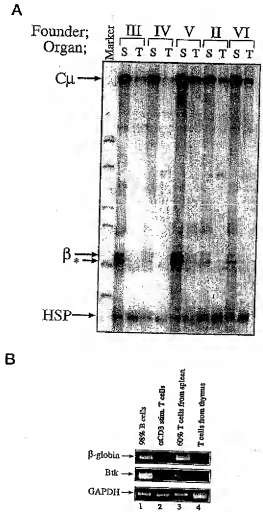

FIGURE 4. Expression of the transgene is restricted to B lineage cells. *A*, RPA with RNA prepared from spleen (S) and thymus (T) from different founder lines as marked above. Indicated by arrows are the specific transcripts for the Cμ (Cμ) and HSP70 controls (HSP), as well as that of the transgene (β). *B*, RT-PCR analysis from MACS-sorted B and T cells. Indicated are the PCR-amplified β-globin product, the Btk product, and the GAPDH product.

3′ region and copy-dependent transgene expression

To firmly evaluate whether the HS1–4 enhancers possessed LCR properties, as described in cell lines (11), we examined the expression of the transgene in relation to the copy number. Copy numbers were determined by Southern blot analysis using a hybridization probe covering the β-globin gene to the *Eco*RI site and the complete V_H promoter. The same blot was subsequently hybridized with a probe specific for HS4 as internal control (data not shown). Data representing the corrected copy number were plotted (Fig. 5*A*) and shown to range from 2–3 (founder I) to 80–82 (founder V) copies. Transgene expression levels were analyzed by RPA and compared with an internal standard (HSP70). Fig. 5*B* shows the relative expression of the β-globin reporter gene corrected by the expression of HSP70 and represents the mean value of three independent experiments. Although these experiments show an overall tendency for copy dependence, there is no strict correlation. Particularly, two founder lines (II and IV) with high copy numbers gave rise to only low levels of expression. Furthermore, even though the expression of the transgene was increased in all founders proportionally, upon stimulation of splenic cells with LPS for 72 h in vitro, we did not obtain copy number-dependent expression (data not shown).

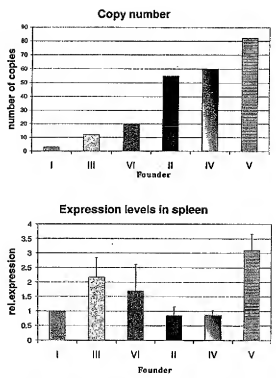

FIGURE 5. Expression of the transgene is not copy dependent. *a*, Copy numbers of the transgenic founder lines. Copy numbers of the individual founder animals were calculated from the intensity of the bands, quantified by PhosphorImager. Copy numbers were corrected by an internal control and plotted against the number of the founder line. *b*, Expression levels of the transgene measured in spleen. The intensity of the specific transcript (measured by PhosphorImager quantification) of the transgene was divided by the intensity measured from the HSP70 transcript and plotted as relative expression against the number of the founder line. Data represent the mean values of three individual experiments.

Discussion

Here we show that a V_H promoter-β-globin reporter gene linked to the HS 1–4 of the IgH locus exhibits B cell-specific expression, thus reproducing the endogenous expression pattern of IgH genes. Using RT-PCR analysis, we show that the transgene is expressed only in splenic B cells or impure T cell fractions that contained residual amounts of B cells, but not in pure T lymphocyte preparations from the thymus or anti-CD3-stimulated splenic T lymphocytes. This is the first time that the endogenous expression pattern of the IgH genes is fully reproduced in a transgenic mouse model. Studies on single enhancer elements in transgenic animals have failed to reconstruct the endogenous expression pattern of the Ig genes, and, in all instances, aberrant expression in other organs have been observed additionally to the spleen. Transgenic mice harboring a V_H promoter/intronic Eμ enhancer-linked reporter gene will be predominantly expressed in lymphoid cells (B and T) but with detectable expression in other organs (20, 22). This expression pattern was found even in the presence of the matrix attachment regions flanking the Eμ enhancer, which have been shown to be important for enhancer activity as well as chromatin accessibility (23, 24). Similarly, a V_H/HS1,2 linked-reporter gene can direct preferential expression to both B and T lineage cells (13). Interestingly, addition of HS3a, HS3b, and HS4 enhancers in the pV_H-LCR construct abrogates the aberrant expression in thymocytes. At present, we do not know the underlying molecular mechanism that directs correct B cell-specific gene expression. One possibility may be that different transcription factors will bind to the enhancer elements forming, a closed structure that could prevent a nonwarranted interaction with other proteins. In the situation with single enhancer elements, such elements would be more accessible to be *trans*-activated by additional transcription factors and perhaps interact with other *cis*-acting elements. Alternatively, active repression could be responsible for the down-regulation of expression in T lymphocytes. Recently, a protein binding to a conserved site in the HS3a and HS3b has been described that forms a complex with small Maf proteins. Bach2 is exclusively expressed at early stages of B cell differentiation and has been suggested to act as a repressor on the IgH 3′ enhancer region (25). A similar protein could account for the repression of the IgH genes in thymocytes.

In a study employing a stably transfected human B cell line the integration of a *c-myc* gene under the control of HS1,2,3b,4 was shown to result in a copy number-dependent and position-independent expression (11). To further assess this issue, we have analyzed the β-globin transgene under control of the HS1–4 in transgenic animals. Although high levels of integration-independent transgene expression in all founders were observed, there was not a strict copy number-dependent expression. We can, of course, not exclude that transgene copies, in the high copy number animals, are transcriptionally inactive. However, fluorescence in situ hybridization (FISH) analysis demonstrated that none of the founders had the transgene integrated in an area of heterochromatin (data not shown). This discrepancy between previous data in cell lines (11) and our study may be explained by the fact that the stably transfected clones are drug selected. Only clones with a certain expression level of the selection marker will be chosen and thereby bias the analysis. On the contrary, no selection pressure is installed on transgenic mice, and all founder lines were analyzed. Similar observations have also been made in the analysis of the β-globin locus or the Eμ enhancer (26, 27). The construct in the plasmacytoma study did not contain HS3a (11), but it appears unlikely that this enhancer alone would account for the difference observed in the present study. The data presented here do not give support for the proposed model of the 6-kb minilocus of the 3′ HS1–4 acting as an LCR in a strict sense. Additional elements may be missing in our construct for the completeness of the LCR. The full palindromic structure of the 30-kb endogenous locus centered at the HS1,2 was not reproduced in our animals; in particular, the inverted repeats flanking HS1,2, which were shown to significantly increase the activity of HS1,2 in plasma cells, were incomplete (28) and may contain elements necessary for a copy number-dependent expression. Alternatively, but not mutually exclusively, it is tempting to speculate on a model of a split LCR composed of the Eμ enhancer and the 3′ HS1–4 that surround the Ig genes (see Fig. 1). In the endogenous locus, a cooperation between the intronic and 3′ enhancer elements with the V_H promoter ensures the correct spatial and temporal expression of Ig genes. The absence of Eμ and its flanking matrix attachment regions may have influenced the LCR function in our animals. Such a model is further supported by the finding that Eμ together with HS4 is active in early B cell development. Both 5′ and 3′ elements may therefore be necessary to border and insulate the Ig locus, keeping it in an open chromatin conformation. The pV_H-LCR construct described here has the unique ability to direct B lymphocyte-specific expression, which indicates that the 3′ regulatory region certainly plays a role in the control of Ig gene expression. The proposed model of a split LCR guiding IgH gene expression is very important to test since such experiments will be instrumental not only to learn about Ig-gene expression but also to further our understanding on the mechanisms of LCR.

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Generation of heavy-chain-only antibodies in mice

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We have generated transgenic mice containing hybrid llama/human antibody loci that contain two llama variable regions and the human D, J, and Cμ and/or Cγ constant regions. Such loci rearrange productively and rescue B cell development efficiently without LC rearrangement. Heavy-chain-only antibodies (HCAb) are expressed at high levels, provided that the CH1 domain is deleted from the constant regions. HCAb production does not require an IgM stage for effective pre-B cell signaling. Antigen-specific heavy-chain-only IgM or IgGs are produced upon immunization. The IgG is dimeric, whereas IgM is multimeric. The chimeric HCAb loci are subject to allelic exclusion, but several copies of the transgenic locus can be rearranged and expressed successfully on the same allele in the same cell. Such cells are not subject to negative selection. The mice produce a full antibody repertoire and provide a previously undescribed avenue to produce specific human HCAb in the future.

immunoglobulin rearrangement | transgenic

Conventional antibodies contain two heavy and light chains (LC) coded for by heavy and LC loci. B cell development and antibody production starts in the bone marrow (BM) by heavy chain (IHC) VDJ recombination and expression of IgM associated with a surrogate LC on the cell surface. In a second round of recombination, one of the LC rearranges in pre-B cells. If successful, the B cells undergo selection, affinity maturation, and switching to different HC constant regions to result in B cells, which express tetrameric antibodies of different isotypes (IgA, IgG, and IgE). Normally absence of HC or LC expression leads to arrest of B cell development. However, some species produce HC-only antibodies (HCAb) as part of their normal B cell development and repertoire. The best-known HCAb (i.e., no LC) are IgG2 and IgG3 in camelids (1). They undergo antigen-mediated selection and affinity maturation, and their variable domains are subject to somatic hypermutation (2, 3). HCAb are thought to recognize unusual epitopes, such as clefts on the antigen surface (4). The first domain of the constant region, CH1, is spliced out because of the loss of a consensus splice signal (5, 6). CH1 exon loss also has been described in other mammals, albeit associated with disease, e.g., in mouse myelomas (7) and human HC disease (HCD) (8–10).

Camelid HCAbs contain a complete VDJ region. Its size, stability, specificity, and solubility have generated considerable biotechnological interest. The antigen-binding site, a single-variable domain (VHH), resembles VH of conventional Abs. However, differences in FR2 and CDR3 prevent VHH to pair with a variable LC, whereas hydrophilic amino acids provide solubility (11). HCAb of the IgM class have not been found in camelids, suggesting that the IgM+ stage of HCAb formation is very transient and/or circumvented.

Murine NSO myeloma cells can express a rearranged camelid VHH-γ2a gene (12) and, recently, the same gene was expressed in transgenic mice (13). Here, we describe transgenic mice containing various nonrearranged chimeric HCAb loci and show they rearrange properly, result in allelic exclusion, efficiently rescue B cell development, and undergo class switch recombination and affinity maturation. They generate functional HCAbs after antigenic challenge, providing a previously undescribed way of producing human HCAb when the llama VHH regions are replaced with soluble human VH.

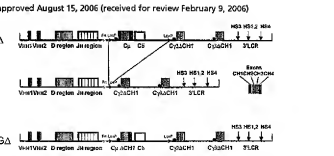

Fig. 1. The transgenic loci. Two llama VHH exons are linked to the human HC diversity (D) and joining (J) regions, followed by the C_μ, C_δ, $C_\gamma 2$, and $C_\gamma 3$ human genes and human HCIg 3′ LCR. The different constant region exons are shown in different colors (see *Middle Right Inset*). CH1 (red) was deleted from $C_\gamma 2$ and $C_\gamma 3$ genes in constructs MGA and GA and also from C_μ in construct MΔGA. LoxP sites (in red) enable removal of C_μ and C_δ genes by cre recombination. The Frt site (in green) enables the generation of a single copy from a multicopy array by Flp recombination.

Results

The CH1 Splice Mutation Is Insufficient for Exon Skipping in the Human HC Locus. It is not known whether the generation of HCAb (IgG2 and 3) in camelids needs an IgM+ stage. Hence, we made two hybrid chimeric loci, one locus (MGS) with human C_μ, C_δ, $C_\gamma 2$, and $C_\gamma 3$ constant regions and one with only $C_\gamma 2$ and $C_\gamma 3$ (GS; Fig. 7, which is published as supporting information on the PNAS web site) in a μMT background (14). μMT animals do not produce surface IgM and have a block in B cell development at the pre-B cell stage. The C_γ regions first were mutagenized to contain the camelid CH1 splice mutation (5). GS was generated because of later reports showing that μMT mice produce some IgG, IgA, and IgE in the absence of membrane IgM (15–17), suggesting some B cells develop without IgM surface expression. Instead of mutating human VH domains to improve solubility (18, 19), two llama VHHs were introduced. Camelid VHH contain characteristic amino acids at positions 42, 49, 50, and 52 (20, 21). VHH1 contained these four, but VHH2 had a Q instead of an E at 49. The locus contained all of the human HC D and J regions and the locus control region (LCR) (Fig. 7). Surprisingly, the splice mutation gave incorrect CH1 exon skipping in mice and no chimeric Ig expression (Fig. 7).

Author contributions: R.J., F.G., and D.D. designed research; R.J., S.D., R.W.H., A.v.R., and D.D. performed research; G.P. and J.K.-a.S. contributed new reagents/analytic tools; R.J., S.D., R.W.H., G.P., F.G., and D.D. analyzed data; and F.G. and D.D. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: BM, bone marrow; HC, heavy chain; HCAb, HC-only antibody; LC, light chain; sdAb, single-domain antibody.

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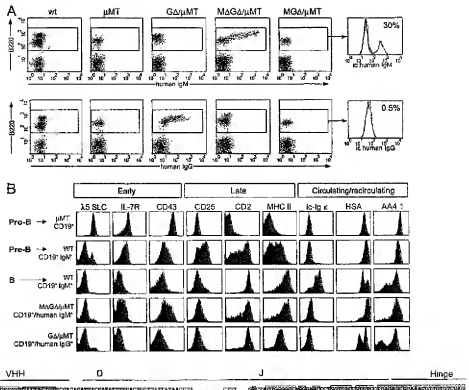

Fig. 2. Flow cytometric analysis of B cells of wt, μMT, MGΔ/μMT, MΔGΔ/μMT, and GΔ/μMT mice in BM. (A) Lymphoid cells were gated on forward and side scatter. Surface expression of B220 and chimeric IgM or IgG is shown as dot plots. For MGΔ/μMT, the B220$^+$ fraction was gated and analyzed for the expression of intracellular (i) chimeric Ig μ and γ H chains, displayed as histogram overlays (red lines), with background stainings of B220$^+$ cells from μMT mice (black lines) as controls. The % of positive cells is indicated. (B) MΔGΔ or GΔ transgenes rescue pre-BCR and BCR function. Shown are the expression profiles of the indicated markers in total CD19$^+$ fractions from μMT mice (pro-B cells), in CD19$^+$ surface IgM$^-$ fractions (pro-B/pre-B cells), and CD19$^+$ surface IgM$^+$ fractions (B cells) from WT, MΔGΔ μMT, and GΔ μMT mice. Ic-Ig κ, intracellular Ig κ LC. Flow cytometric data are displayed as histograms representative of 3–8 animals examined in each group. (C) Sequence alignment of BM cDNA showing VDJ recombination. Sequences are from GΔ. Green shows sequence identity.

Chimeric Loci Lacking a Human CH1 Region. The CH1 splice problem was solved by generating three new constructs (Fig. 1), all containing Cγ2 and Cγ3 with CH1 deleted, one with Cμ and Cδ (MGΔ), one without Cμ and Cδ (GΔ), and one with CH1 deleted Cμ (MΔGΔ). Three MGΔ, six GΔ, and four MΔGΔ transgenic mouse lines with one to five copies were obtained in a μMT background. Mice with different copy numbers gave the same results.

GΔ and MΔGΔ rescue B cell development. GΔ and MΔGΔ, but not MGΔ, rescued B cell development in a μMT background. The rescue of B220/CD19 cells was between 30% and 100% in different lymphoid compartments independent of copy number (Fig. 2*A* and Table 1). The MΔGΔ mice contain human IgM-producing cells in the BM absent in WT or μMT mice. Appropriately, they have not switched class because chimeric IgG is absent. The GΔ mice contain only chimeric IgG$^+$ B cells. The MGΔ mice contain very few B cells expressing cell-surface chimeric Ig, but interestingly, 30% of the BM B220 cells express intracellular IgM, but not IgG (Fig. 2*A*). The MGΔ, but not the MΔGΔ and GΔ (data not shown), express mouse Ig LC (see Fig. 5*G*). Thus, the Cμ and Cγ genes are expressed, and absence of CH1 is crucial for surface-expressed HCAb.

HCAb replace mouse (pre-)BCR in the BM. During progression of large cycling into small resting pre-B cells, specific surface markers are down-regulated in a pre-BCR-dependent manner (22). To test whether chimeric HCAbs functionally replace the pre-BCR, various markers were analyzed. Pro-B cells express high cytoplasmic SLC, IL-7R and CD43, which are down-regulated upon pre-BCR expression and absent in mature B cells (Fig. 2*B*).

MΔGΔ/μMT or GΔ/μMT chimeric Ig$^-$ B cells are SLC- and

Table 1. Percent of B220$^+$/CD19$^+$ cells in total population of nucleated cells

Cell type	WT	GΔ (~5 copies)	GΔ (single copy)	MΔGΔ
BM	10.80 ± 2.09	5.94 ± 1.44	4.93 ± 1.79	6.06 ± 1.53
Spleen	41.80 ± 6.05	32.14 ± 9.46	28.70 ± 8.70	33.95 ± 3.74
Blood	43.72 ± 2.50	16.00 ± 5.68	16.01 ± 3.76	9.25 ± 3.24
Peritoneum	21.32 ± 3.30	22.85 ± 6.71*	22.30 ± 7.29*	21.21 ± 14.42

Mice were 14–20 weeks old. Numbers of mice analyzed are 5–11 per mouse line with the exception of two peritoneal cell measurements, where calculations are based on two samples (marked by asterisks).

IL-7R-low, indicating that the chimeric HC IgG and IgM receptors function as a pre-BCR in down-regulating SLC and IL-7R. CD43 persists in MΔGΔ (not in GΔ) mice, perhaps due to increased B-1 B cell differentiation. CD2 and MHC class II are induced normally. The levels of the IL-2R/CD25, transiently present in pre-B cells, are very low on mature MΔGΔ or GΔ/μMT B cells as in WT (Fig. 2*B*). ic Igκ was absent in mature MΔGΔ or GΔ/μMT B cells (Fig. 2*B*) and was not induced in BM cultures upon IL-7 withdrawal after IL-7$^+$ culture (data not shown). Finally, the chimeric HCAb$^+$ B cell populations in MΔGΔ or GΔ mice consisted of cells generated in the BM (HSA^{high} and AA4.1/CD93^{high}) and cells matured in the periphery that are recirculating (HSA^{low} and CD93^{low}) as in wild type.

Thus, chimeric HCAb IgG and IgM function as (pre-)BCR with respect to developmentally regulated markers. IgL chain is not induced (see below). Both VHHs are used for VDJ recombination, CH1 is absent and, importantly, CDR3 shows a large diversity (Fig. 2*C*).

Multiple Rearrangements and Allelic Exclusion. MΔGΔ and GΔ hybridomas were made after immunization. Particularly, the five-copy GΔ line1 could have more than one rearrangement. Of the five different five-copy hybridomas, one rearranged one in frame copy; two hybridomas had two rearrangements, each with one out of frame; one hybridoma had two in-frame rearrangements; and one hybridoma had four rearrangements, with two in frame.

Two express two productive mRNAs (mass spectrometry confirmed the secreted HCAbs matching the cDNA; data not shown). We also carried out DNA fiber FISH on a hybridoma with one rearrangement and normal FISH on one with four rearrangements by using an LCR probe detecting each copy and a probe between VHH and D detecting only nonrearranged copies (Fig. 3 *A–E*). Control cells showed five copies plus half a copy at each end (Fig. 3*A*), in agreement with Southern blots (data not shown), whereas the hybridomas show one and four rearranged copies, respectively (Fig. 3 *B–E*). Thus, multiple copies can rearrange successfully on the same allele.

Moreover B220/CD19-positive BM cells of GΔ line1 transgenic mice in a WT background were analyzed for the expression of transgenic IgG and mouse IgM. Clearly, the GΔ B cells express either mouse Ig or chimeric Ig (Fig. 3*F*), showing allelic exclusion. *Splenic B cells.* Splenic B cell subpopulations were analyzed by using CD21/CD23 (Fig. 4*A*). GΔ CD21^{low}CD23^{low} immature B cells were

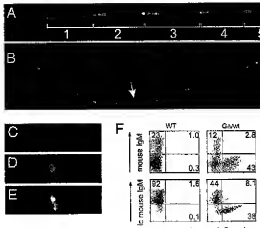

Fig. 3. DNA FISH and allelic exclusion of a five-copy chimeric GΔ locus. (*A*) Stretched chromatin fiber from lung cells of GΔ line1 carrying five intact copies (1–5) of the GΔ locus, flanked by half of a locus containing the LCR (red) and half of a locus containing VHH to J region (green). (*B*) Stretched chromatin fiber FISH of a hybridoma (G20) derived from GΔ line1 B cells where one copy has rearranged (white arrow). (*C*) Nonstretched DNA FISH of hybridoma T1 with the LCR probe (red). (*D*) Same as C with a probe between VHH and D (green). (*E*) Overlay of C and *D*. Note that T1 has four rearrangements visible because of the loss of four green signals with no loss of red signals. (*F*) Allelic exclusion in GΔ mice. Flow cytometric analysis of murine surface or intracellular (ic)μ H chain and chimeric IgG on total BMCD18$^+$ cells from GΔ transgenic mice in a WT background and a nontransgenic WT control mouse displayed as dot plots. The % of cells within the quadrants is indicated. The average extracellular and intracellular double expressors after subtraction of the background were 1.5% and 5.8%, respectively (n = 9).

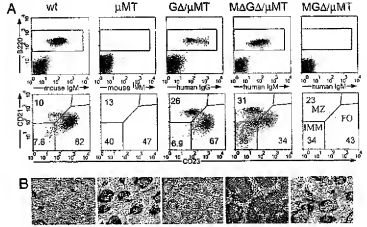

Fig. 4. B cell populations in the spleen of WT, μMT, GΔ, MΔGΔ, and MGΔ mice. Data shown are representative of 4–8 mice examined in each group. (*A Upper*) FACS data of spleen cells, stained for mouse IgM, chimeric IgG, chimeric IgM versus B220. (*A Lower*) Flow cytometric analysis of B cell populations in spleen. Lymphoid cells were gated on forward and side scatter. Surface expression of B220 and the indicated Ig (*A Upper*) or the CD21/CD23 profile is displayed as dot plots and the % of cells within the indicated gates are given. CD21^{low}CD23^{low}, immature B cells; CD21$^+$CD23$^+$, follicular B cells; CD21^{high}CD23^{low}, marginal zone B cells. (*B*) Histology of the spleen of WT, μMT, GΔ/μMT, MΔGΔ/μMT, and MGΔ/μMT mice. Immunohistochemical analysis is shown after 5-μm frozen sections were stained with αB220 (blue) for B cells and αCD11c/N418 (brown) for dendritic cells. Arrow indicates a small cluster of B cells in MGΔ spleen. (*C*) Sequence alignment of Peyer's patches cDNA showing that the transgenic locus undergoes hypermutation in the CDR1 and 2 regions. Sequences are from the transgenic locus GΔ with a CH1 deletion.

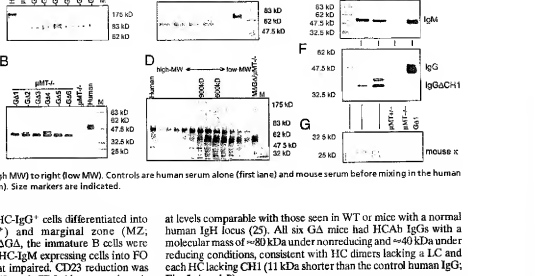

Fig. 5. Prot G or concanavalin purified serum samples of six different GΔ lines (*A* and *B*), four MAGΔ lines (*C*), and two MGΔ lines (*E–G*) in the μMT background run under nonreducing (*A*) and reducing conditions (*B–G*). The size of the chimeric IgG (*B* and *F*) and IgM (*C* and *D*) is consistent with a CH1 deletion and absence of LC. Mouse κ LC were normal size (*G*). Human serum was used as a positive control. (*D*) Superose 6 size fractionation of MAGΔ serum after mixing in a human IgM control under nonreducing conditions. Each fraction was analyzed by gel electrophoresis under reducing conditions. Fractions collected of the column are from left (high MW) to right (low MW). Controls are human serum alone (first lane) and mouse serum before mixing in the human IgM control serum (lane MAGΔ serum). Size markers are indicated.

in normal ranges, and chimeric $HC\text{-}IgG^+$ cells differentiated into follicular (FO, $CD21^+CD23^+$) and marginal zone (MZ, $CD21^{high}CD23^{low}$) B cells. In MAGΔ, the immature B cells were increased, i.e., differentiation of HC-IgM expressing cells into FO and MZ B cells appear somewhat impaired. CD23 reduction was accompanied by increased CD43 and CD5 (data not shown), indicative of differentiation into B-1 B cells. The few chimeric IgM expressing B cells (also expressing mouse LC, see Fig. 5) in MGΔ mice had a FO/MZ distribution similar to MAGΔ mice.

Spleen architecture in MAGΔ and GΔ, but not MGΔ mice, is normal (Fig. 4*B*). As in wild type, germinal centers in B cell follicles are formed (data not shown) during T cell-dependent responses that in GΔ mice contain chimeric IgG^+ cells. We confirmed hypermutation of the HCAb by cDNA analysis from B cells present in Peyer's patches. (Fig. 4*C*). Both VHHs are used. Thus, in GΔ and MAGΔ mice, immature B cells migrating from BM differentiate into spleen FO and MZ B cells and undergo somatic hypermutation upon antigen challenge.

Single-copy loci rescue efficiently and CH1 absence is essential. The GΔ line1 mice (Table 1) had five copies and, hence, the efficient rescue was related possibly to the copy number of the locus. A single-copy line generated from the GΔ line1 through breeding with a FlpeR line (23) gave the same B cell rescue (Table 1; Fig. 8, which is published as supporting information on the PNAS web site).

Confirmation that a single copy of the locus is sufficient for rescue and that a CH1 region is inhibitory was obtained by cre-mediated deletion of the Cμ and Cδ from MGΔ line 3, resulting in a single-copy GΔ line (Fig. 8). This locus now rescues B cell development like the other GΔ lines. Thus, a CH1 region in Cμ inhibits B cell rescue, and copy number is not important.

Mouse Light Chains Do Not Rearrange in MAGΔ and GΔ Mice. Murine LC were absent in the MAGΔ and GΔ mice by Western blots (data not shown, but see Fig. 2*B* and *SA*) or FACS, suggesting that the LC genes do not rearrange as confirmed by comparing the Igκ locus germ-line signals in sorted splenic B220$^+$ cells and liver DNA by Southern blots (Fig. 9, which is published as supporting information on the PNAS web site). Mouse LC remain in a germ-line configuration. In contrast, LC are present in the few chimeric Ig^+ cells in the MGΔ/μMT mice (see Fig. 5*G*).

Thus, the chimeric HCAb expression in early B cell development in BM fails to signal for LC rearrangement. In this respect, HCAb lacking CH1 mimic a BCR rather than a pre-BCR, probably because of a failure to bind pseudo-LC (24).

Serum analysis. Chimeric IgM was present in MAGΔ and chimeric IgG in both MAGΔ and GΔ serum. In nonimmunized adults, the chimeric IgM (≈50 μg/ml) and IgG (200–1,000 μg/ml) are present at levels comparable with those seen in WT or mice with a normal human IgH locus (25). All six GΔ mice had HCAb IgGs with a molecular mass of ≈80 kDa under nonreducing and ≈40 kDa under reducing conditions, consistent with HC dimers lacking a LC and each HC lacking CH1 (11 kDa shorter than the control human IgG; Fig. 5 *A* and *B*).

The MAGΔ serum had multimeric HC-IgM. Under reducing conditions (Fig. 5*C*), all four lines had IgM with the molecular mass of a human IgM after subtraction of CH1. Serum also was fractionated (Fig. 5*D*, horizontal fractions) under nonreducing conditions, and each fraction was analyzed under reducing conditions (Fig. 5*D*, vertical lanes). When compared with the human pentameric 900-kDa IgM, the transgenic IgM is 600 kDa, consistent with a multimer lacking LC and CH1. Thus, MAGΔ or GΔ mice produce multimeric IgM and/or dimeric IgG.

MGΔ Mice. Some clustered B220-positive cells (<1% of the WT) are seen in MGΔ/μMT spleens (Fig. 4*B*), and serum chimeric IgM and IgGs were detected only after purification (Fig. 5 *E* and *F*). The IgM in these mice was normal size, whereas the IgGs are shorter because of CH1 deletion. Interestingly mouse κLC, presumably associated with the chimeric IgM, also were detected (Fig. 5*G*).

Immunization. The GΔ/μMT mice were immunized with *Escherichia coli* hsp70, DKTP (*Diphteria* toxoid, whole cell lysate of *Bordetella pertussis*, *Tetanus* toxoid, and inactivated poliovirus types 1–3), and rtTA (26), the MAGΔ mice with human TNFα. Antibodies were isolated from hybridomas or single-domain Ab (sdAb) phage display libraries.

Sequencing (Fig. 6*A*) showed that both IgG2 (seven of eight) and IgG3 (one of eight) were produced (the sdAb were isolated from a IgG2 library). Different D and J regions were used. When comparing all 14 antibodies, it was evident that all J regions are used, but as in humans, JH4 is used most frequently. Surprisingly, all antibodies had VHH2 (with a Q rather than E at position 49; ref. 20). Clearly CDR3 provides most diversity (27). It varies between 10 and 20 aa (average of 13.6 aa), as in llamas and humans (28, 29). Although not at high frequency, the VHH were hypermutated. The 3α-hTNFα antibodies (Fig. 5) had different hypermutations in the CDR2 region.

The HCAb are functional in regular assays as hybridoma supernatants and bacterial periplasmic fractions of sdAbs (Fig. 6). All were positive in ELISAs and in antigen detection on Western blots (Fig. 6*B*). We also tested the α-rtTA IgG in immunocytochemistry in a rtTA$^+$ cell line (Fig. 6 *C* and *D*). The avidity of a number of the antibodies was high, although some were low. For example, binding studies of the α-rtTA antibody used in the immunocytochemistry

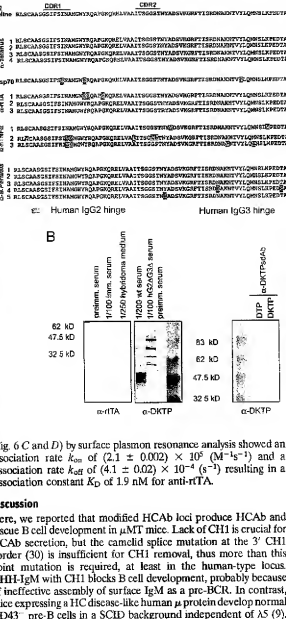

Fig. 6. Antibody properties. (*A*) Sequences of monoclonal antibody cDNAs specific for tetanus toxoid: HSP70, rtTA, and human TNFα. The top sequence is the germ-line VHH2 sequence. The CDR 1, 2, and 3 and hinge regions are indicated above the sequence. Different isotypes and classes are indicated by different colors on the right. The *J* regions that are used are indicated on the right. (*B*) Examples of Western blots by using the different HCAb (hybridomas, sera, and sdAb). (*B Left*) anrtTA serum and hybridoma medium, diluted 1/100 and 1/250. (*B Center*) αDKTP serum from WT and GA mice diluted 1/200 and 1/100. (*B Right*) α *B. pertussis* sdAb against vaccine containing *B. pertussis* antigen (DKTP) or lacking it (DTP, because we were unable to purchase purified *B. pertussis* antigen). (*C* and *D*) Immunostaining of a Tet^on^ cell line with a marker plasmid responding to rtTA by expressing a marker in cytoplasm (44). (*C*) Nuclei expressing rtTA (green). (*D*) Doxycycline-induced expression of the marker (red) in response to rtTA and DAPI nuclear staining (blue).

(Fig. 6 *C* and *D*) by surface plasmon resonance analysis showed an association rate k_{on} of $(2.1 \pm 0.002) \times 10^5$ $(M^{-1}s^{-1})$ and a dissociation rate k_{off} of $(4.1 \pm 0.02) \times 10^{-4}$ (s^{-1}) resulting in a dissociation constant K_D of 1.9 nM for anti-rtTA.

Discussion

Here, we reported that modified HCAb loci produce HCAb and rescue B cell development in μMT mice. Lack of CH1 is crucial for HCAb secretion, but the camelid splice mutation at the 3′ CH1 border (30) is insufficient for CH1 removal, thus more than this point mutation is required, at least in the human-type locus VHH-IgM with CH1 blocks B cell development, probably because of ineffective assembly of surface IgM as a pre-BCR. In contrast, mice expressing a HC disease-like human μ protein develop normal $CD43^-$ pre-B cells in a SCID background independent of λ5 (9). Truncated IgM expressed on the B cell surface without L chains mimics pre-BCR signaling through self-aggregation (10).

Normally BiP chaperones the folding and assembly of antibodies by binding CH1 until it is replaced by (surrogate) LC (31). Although VpreB1 and 2 can bind normal IgM in the absence of λ5 (32), our results suggest that transgenic μ HC pairing with the Vpre protein does not take place when a CH1-containing μ HC is linked to a VHH, which would lead to a failure of B cell development (33). The MGS and MGA transgenic mice containing a CH1 would be able to form some pre-BCR-like complex that may lead to signaling, causing some expansion and developmental progression and explain why 30% of the B220-positive cells in BM have intracellular IgM (Fig. 2*A*). The few matured B cells in spleen of these mice may be explained by the recently described novel receptor complex lacking any SLC or LC (34).

When IgM is removed and $C\gamma 2$ and 3 lack CH1, there is rescue of B cell development, showing that IgG can functionally replace IgM. IgG1 expression from the pro-B cell stage onwards, was shown to substitute for IgM in $Rag2^{-/-}$ B cell development (35). Recently, it also was shown that a pretearranged camelid IgG2a partially rescues B cell development in one transgenic line in a μMT (and a $C\lambda^{-/-}$) background (13). In our case, IgM or IgG lacking CH1 rescue B cell development in 10 of 10 independent lines. Moreover, we see no LC rearrangement and conclude that LC are not required for further B cell differentiation. The difference in our results and those of Zou *et al.* (13) may be explained by the level of expression of the locus (and, thus, signaling) because of the LCR on our constructs. Our results confirm that truncated μ HCAb lacking CH1 (24) or VH and CH1 (36) cannot associate with SLCs and fail to activate κ gene rearrangement.

Interestingly, one or more HC rearrangements occur in multicopy loci (Fig. 3). Two of the hybridomas, originating from two separate splenocytes gave two productive HCAb, confirming that expression of two antibodies in one B cell is not toxic (37). However, the prediction (37) that they would loose in competition with single antibody-producing cells under antigen challenge is not borne out by finding two double antibody-expressing cells of five hybridomas.

The (multicopy) locus is subject to and exerts allelic exclusion in WT background, because BM cells express either mouse or chimeric cell surface Ig. Few BM cells expressed both on the surface (Fig. 3*F*). Interestingly, a five-copy GA WT mouse has two mouse alleles with one Ig locus each and one allele with five chimeric HCAb loci. If alleles are chosen, there should be more mouse than chimeric Ig expression, and if genes are chosen, there should be more chimeric than mouse Ig expression. In fact, mouse Ig is

expressed more often (44/38; Fig. 3*F*). Ignoring possible deviations from the random V use and a possible position effect on the transgenic locus, suggesting that the first choice is one of alleles.

Normally, a productive rearrangement down-regulates recombination to prevent rearrangement of the other allele. However, the multiple transgenic copies, when rearranged, exclude the mouse endogenous locus, but fail to exclude further rearrangement on the same open locus before RAG down-regulation. This process may involve a spatial component ("compartment"), in that the time before the RAGs are down-regulated would be sufficient to rearrange another gene in the locus because it would be in close proximity. The observation that other species with multiple loci on the same chromosome have more cells expressing two Abs (38) supports this argument. Alternatively multiple rearrangements may take place at the same time.

Importantly, we show that HCAb loci can be expressed successfully in mice. Antigen challenge results in antigen-specific chimeric HCAb of different classes (dependent on locus composition) expressed at levels comparable with WT or conventional human IgH transgenic mice (25). Only two VHHs were used, yet antibodies with diverse specificity were isolated successfully to almost all of the totally unrelated proteins we tested, demonstrating the efficiency and efficacy of diversity generated by CDR3 (27). Thus, having V(D)J recombination and *in vivo* selection provides an advantage over antibodies of fragments thereof from synthetic libraries. Hybridomas containing HCAb with a human effector function are generated easily. They can be used also for direct cloning and expression of sdAb, which can alternatively also be derived by phage display.

Thus, these mice open up new possibilities to produce human HCAb for clinical or other purposes, particularly in light of the evidence (4) that HCAbs may recognize "difficult" epitopes such as enzyme active sites. The restricted number of VH may explain why not all antigens were recognized; the polio and *Diphteria* proteins gave no response in GΔ mice, whereas WT control mice did (data not shown). Surprisingly, all antibodies had VHH2 lacking a conserved amino acid (39) at position 49 in contrast to VHH1 that has one and should be more soluble. Perhaps, VHH1 expression results in negative selection.

The addition of more VHs should lead to an even broader repertoire. Whilst it is preferable to avoid multiple copies on a single allele, it would be advantageous to have multiple alleles with a single copy of different VH regions to increase diversity. In such new loci, one can use either normally occurring (human) VH or VH engineered for increased solubility (18).

In conclusion, we show that antigen-specific HCAb of potentially any class can be produced in mice. By introducing soluble human VH domains in the locus, this technology allows the production of fully human HCAb of any class or fragments thereof in response to antigen challenge for use as therapeutic agents in man. By using different vertebrate loci, our technology also allows for production of antibodies from any vertebrate for use as reagents, diagnostics, or for the treatment of animals.

Materials and Methods

A standard genomic cosmid library was made from *Lama glama* blood. Two germ-line VHHs were chosen with hydrophilic amino acid codons at positions 42, 50, and 52 according to ImMunoGeneTics numbering (40), one with and one without a hydrophilic amino acid at 49. One is identical to IGHV1S1 (GenBank accession no. AF305944), and the other has 94% identity with IGHV1S3 (GenBank accession no. AF305946). PAC clone 1065 N8 contained human HC D and J regions, Cμ and Cδ, and clone 1115 N15 contained C γ3 (BACPAC Resource Center, Oakland, CA). Bac clone 11771 (Incyte Genomics, Palo Alto, CA) was used to obtain Cγ2 and the HC-LCR (41). Cγ3 and Cγ2 were subcloned separately into pFastBac (Invitrogen, Carlsbad, CA). The point mutation (G to A) (5) or deletion of CH1 was done by recombination (42). Similarly, frt and lox P sites were introduced 5′ to the Cμ switch region, and a second lox P site was placed 5′ to the Cγ2 switch region, resulting in MGS or MGΔ.

GS or GΔ were generated from MGS or MGΔ (Figs. 1 and 7) by cre recombination (43). MΔGΔ was obtained from MGΔ by deletion of the Cμ CH1 region through homologous recombination. The generation of transgenic mice, breeding, and genotyping, RT-PCR, flow cytometry, Ig gene arrangement, DNA FISH analysis, immunization and hybridoma production, sdAB library production and screening, immunocytochemistry, Western blots, gel filtration, and BIAcore measurements are described in *Supporting Methods*, which is published as supporting information on the PNAS web site.

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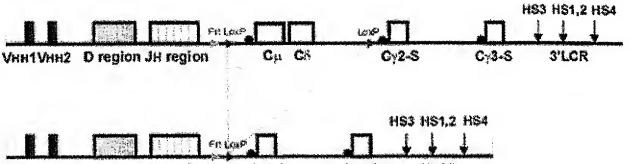

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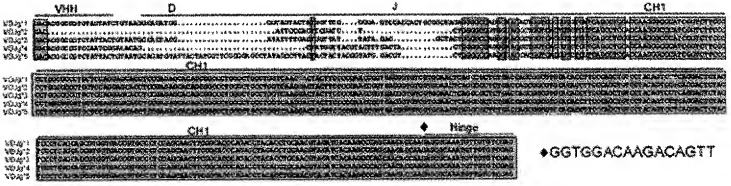
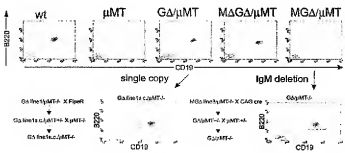


C

	CH1	IVS I
Human cγ2	ACAAGACAGTTG	GTGAGAGG
Human cγ3	ACAAGAGAGTTG	GTGAGAGG
Camel cγ2a (Nguyen *et al.*)	ACAAGAGTGTGG	ATAAGTAG
MGS or GS cγ2	ACAAGACAGTTG	ATGAGAGG
MGS or GS cγ3	ACAAGAGAGTTG	ATGAGAGG
patient (Zhao *et al.*)	ACAAGACAGTTG	GTGGGAGG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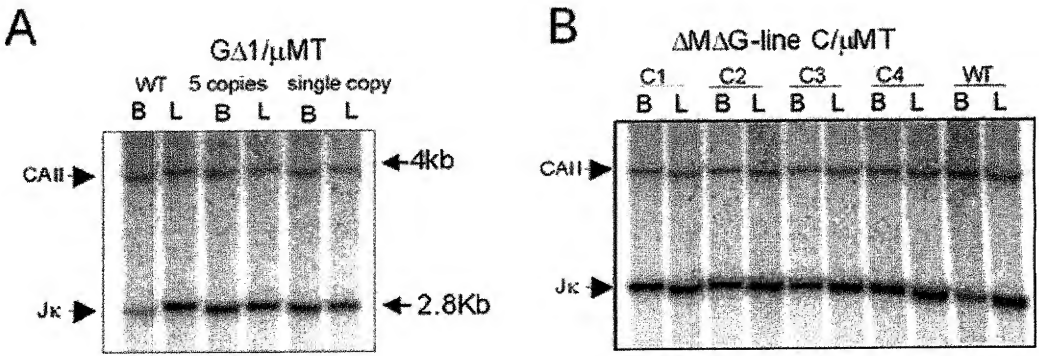
µMT
GΔ/µMT
MΔGΔ/µMT
MGΔ/µMT
B220
CD19
single copy
IgM deletion
CD19
CD19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GΔ1/μMT
WT
5 copies
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B L B L B L
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(54) **Title:** PRODUCTION OF HUMANIZED ANTIBODIES IN TRANSGENIC ANIMALS

(57) **Abstract:** This invention relates to humanized antibodies and antibody preparations produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

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Production of Humanized Antibodies In Transgenic Animals

Field of the Invention

This invention relates to humanized antibodies produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

Background of the Invention

The therapy of infectious diseases caused by bacteria, fungi, virus and parasites is largely based on chemotherapy. However, the emergence of drug-resistant organisms requires the continuous development of new antibiotics. Therapies of patients with malignancies and cancer are also based on chemotherapy. However, many of these therapies are ineffective and the mortality of diseased patients is high. For both infectious diseases and cancer, improved and innovative therapies are needed.

Therapy of steroid resistant rejection of transplanted organs requires the use of biological reagents (monoclonal or polyclonal antibody preparations) that reverse the ongoing alloimmune response in the transplant recipient. The major problem of antibody preparations obtained from animals is the intrinsic immunogenicity of non-human immunoglobulins in human patients. In order to reduce the immunogenicity of non-human antibodies, genetic engineering of individual antibody genes in animals has been proposed. In particular, it has been shown that by fusing animal variable (V) region exons with human constant (C) region exons, a chimeric antibody gene can be obtained. However, this approach may only eliminate the immunogenicity caused by the non-human

Fc region, while the remaining non-human Fab sequences may still be immunogenic. In another approach, human immunoglobulin genes for both, heavy and light chain immunoglobulins have been introduced into the genome of mice. While this genetic engineering approach resulted in the expression of human immunoglobulin polypeptides in genetically engineered mice, the level of human immunoglobulin expression is low. This may be due to species-specific regulatory elements in the immunoglobulin loci that are necessary for efficient expression of immunoglobulins. As demonstrated in transfected cell lines, regulatory elements present in human immunoglobulin genes may not function properly in non-human animals.

Several regulatory elements in immunoglobulin genes have been described. Of particular importance are enhancers downstream (3') of heavy chain constant regions and intronic enhancers in light chain genes. In addition, other, yet to be identified, control elements may be present in immunoglobulin genes. Studies in mice have shown that the membrane and cytoplasmic tail of the membrane form of immunoglobulin molecules play an important role in expression levels of human-mouse chimeric antibodies in the serum of mice homozygous for the human Cγ1 gene. Therefore, for the expression of heterologous immunoglobulin genes in animals it is desirable to replace sequences that contain enhancer elements and exons encoding transmembrane (M1 exon) and cytoplasmic tail (M2 exon) with sequences that are normally found in the animal in similar positions.

The introduction of human immunoglobulin genes into the genome of mice resulted in expression of a diversified human antibody repertoire in genetically engineered mice. In both mice and humans, antibody diversity is generated by gene rearrangement. This process results in the generation of many different recombined V(D)J segments encoding a large number of antibody molecules with different antigen binding sites. However, in other animals, like rabbits, pigs, cows and birds, antibody diversity is generated by a substantially different mechanism called gene conversion. For example, it is well established that in rabbit and chicken, VDJ rearrangement is very limited (almost 90% of immunoglobulin is generated with the 3'proximal VH1 element) and antibody diversity is generated by gene conversion and hypermutation. In contrast, mouse and

human gene conversion occurs very rarely, if at all. Therefore, it is expected that in animals that diversify antibodies by gene conversion a genetic engineering approach based on gene rearrangement will result in animals with low antibody titers and limited antibody diversity. Thus, the genetic engineering of large animals for the production of non-immunogenic antibody preparations for human therapy requires alternative genetic engineering strategies.

Relevant Literature

The use of polyclonal antibody preparations for the treatment of transplant rejection was recently reviewed by N. Bonnefoy-Berard et al., *J Heart Lung Transplant* 1996; 15(5): 435-442; C. Colby et al., *Ann Pharmacother* 1996; 30(10):1164-1174; M.J. Dugan et al., *Ann Hematol* 1997; 75(1-2):41-46. The use of polyclonal antibody therapies for autoimmune diseases has been described by W. Cendrowski, *Boll Ist Sieroter Milan* 1997; 58(4):339-343; L.K. Kastrukoff et al., *Can J Neurol Sci* 1978; 5(2):175-178; J.E. Walker et al., *J Neurol Sci* 1976; 29(2-4):303-309. The depletion of fat cells using antibody preparations has been described by L. De Clercq et al., *J Anim Sci* 1997; 75(7):1791-1797; J.T. Wright et al., *Obes Res* 1995; 3(3):265-272.

Regulatory elements in immunoglobulin genes have been described by Bradley et al. (1999), *Transcriptional enhancers and the evolution of the IgH locus*; Lauster, R. et al., *Embo J* 12: 4615-23 (1993); Volgina et al., *J Immunol* 165:6400 (2000); Hole et al., *J Immunol* 146:4377 (1991).

Antibody diversification by gene conversion in chicken and rabbit has been described by Bucchini et al., *Nature* 326: 409-11 (1987); Knight et al., *Advances in Immunology* 56: 179-218 (1994); Langman et al., *Res Immunol* 144: 422-46 (1993). The generation of mice expressing human-mouse chimeric antibodies has been described by Pluschke et al., *Journal of Immunological Methods* 215: 27-37 (1998). The generation of mice expressing human-mouse chimeric antibodies with mouse derived membrane and cytoplamic tails has been described by Zou et al., *Science* 262: 1271-1274 (1993); Zou et al. *Curr Biol* 4: 1099-1103. The generation of mice expressing human immunoglobulin polypeptides has been described by Bruggemann et al. *Curr Opin Biotechnol* 8(4): 455-8 (1997); Lonberg et al. *Int Rev Immunol* 13(1):65-93 (1995); Neuberger et al., *Nature* 338:

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The production of transgenic chicken has been described by Etches et al., *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and potential applications", *Trends Biotechnol 12:415* (1994); and in WO 200075300, "Introducing a nucleic acid into an avian genome, useful for transfecting avian blastodermal cells for producing transgenic avian animals with the desired genes, by directly introducing the nucleic acid into the germinal disc of the egg".

Agammaglobulinemic chicken have been described by Frommel et al., *J Immunol* 105(1): 1-6 (1970); Benedict et al., *Adv Exp Med Biol* 1977; 88(2): 197-205.

The cloning of animals from cells has been described by T. Wakayama et al., *Nature* 1998; 394:369-374; J.B. Cibelli et al., *Science* 280:1256-1258 (1998); J.B. Cibelli et al., *Nature Biotechnology* 1998; 16:642-646; A. E. Schnieke et al., *Science* 278: 2130-2133 (1997); K.H. Campbell et al., *Nature* 380: 64-66 (1996).

Production of antibodies from transgenic animals is described in U.S. Patent No. 5,814,318, No. 5,545,807 and No. 5,570,429. Homologous recombination for chimeric mammalian hosts is exemplified in U.S. Patent No. 5,416,260. A method for introducing DNA into an embryo is described in U.S. Patent No. 5,567,607. Maintenance and expansion of embryonic stem cells is described in U.S. Patent No. 5,453,357.

The mechanisms involved in the diversification of the antibody repertoire in pigs, sheep and cows are reviewed in Butler, J. E. (1998), "Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals", *Rev Sci Tech 17:43*. Antibody diversification in sheep is described in Reynaud, C. A., C. Garcia, W. R. Hein,

and J. C. Weill (1995), "Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process", *Cell 80:115*; and Dufour, V., S. Malinge, and F. Nau. (1996), "The sheep Ig variable region repertoire consists of a single VH family", *J Immunol 156:2163.*

Summary of the Invention

One embodiment of the present invention provides humanized antibodies (humanized immunoglobulins) having at least a portion of a human immunoglobulin polypeptide sequence.

The humanized antibodies of the present invention are made from transgenic non-human animals genetically engineered to contain one or more humanized Ig loci.

Preferably, the humanized antibodies of the present invention are prepared from transgenic non-human animals which generate antibody diversity primarily by gene conversion and hypermutation, e.g., rabbit, pigs, chicken, sheep, cow and horse. The antibodies can be made by immunizing transgenic animals with a desired antigen such as an infectious agent (e.g., bacteria or viruses) or parts or fragments thereof.

Such humanized antibodies have reduced immunogenicity to primates, especially humans, as compared to non-humanized antibodies prepared from non-human animals. Therefore, the humanized antibodies of the present invention are appropriate for use in the therapeutic treatment of human subjects.

Another embodiment of the present invention provides a preparation of humanized antibodies which can be monoclonal antibodies or polyclonal antibodies. Preferred antibody preparations of the present invention are polyclonal antibody preparations which, according to the present invention, have minimal immunogenicity to primates, especially humans.

A preferred preparation of polyclonal antibodies is composed of humanized immunoglobulin molecules having at least a heavy chain or light chain constant region polypeptide sequence encoded by a human constant region gene segment. More preferably, the variable domains of the heavy chains or light chains of the immunoglobulins molecules are also encoded by human gene segments.

In another embodiment, the present invention provides pharmaceutical compositions which include a preparation of humanized antibodies, and a pharmaceutically-acceptable carrier.

Another embodiment of the present invention provides novel sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating the antibody diversity. In particular, the present invention provides novel nucleotide sequences downstream (3', 3-prime) of the genes coding for Cλ in chickens, Cγ and Cκ in rabbits, Cγ1,2,3 in cows and Cγ1,2 in sheep, as well as novel sequences 5' of rabbit Cγ.

In another embodiment, the present invention provides recombination vectors useful for replacing an Ig gene segment of a non-human animal with the corresponding human Ig gene segment. These vectors include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences are homologous to the flanking sequences of the target animal Ig gene segment.

Preferred recombination vectors are those useful for the replacement of the animal's Ig constant region. For example, recombination vectors useful for replacing the rabbit heavy chain constant region genes are provided. A preferred vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13, or a portion of SEQ ID NO: 12 or SEQ ID NO: 13, a human heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of or SEQ ID NO: 10. Another preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 51, which sequence is characterized as having a human Cγ1 gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

Recombination vectors are also provided useful for replacing the rabbit light chain constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human Cκ linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain Cκ1 gene.

Other recombination vectors are provided which are useful for replacing the chicken light chain constant region genes. A preferred vector contains a nucleotide

sequence as set forth in SEQ ID NO: 57 which is characterized as having a human Cλ2 linked to flanking sequences from the 5' and 3' flanking regions of the chicken light chain Cλ gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

In still another embodiment, the present invention provides transgenic constructs or vectors containing at least one humanized Ig locus, i.e., an Ig locus from a non-human animal or a portion of an Ig locus from a non-human animal wherein the locus or the portion of a locus is genetically modified to contain at least one human Ig gene segment. Such humanized Ig locus has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulins.

One humanized Ig locus provided by the invention is a humanized heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in the humanized heavy chain locus are juxtaposed with respect to each other in an unrearranged, or partially or fully rearranged configuration. A preferred humanized heavy chain locus contains a human constant region gene segment, preferably, Cα or Cγ. A more preferred humanized locus contains multiple V gene segments and at least one human V gene segment, in addition to a human heavy chain constant region segment. The human V gene segment is placed downstream of the non-human V gene segments.

Another humanized Ig locus is a humanized light chain locus which includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed with respect to each other in an unrearranged or rearranged configuration. A preferred

humanized light chain locus contains a human constant region gene segment, preferably, Cλ or Cκ. More preferably, the humanized light chain locus further contains multiple V gene segments and at least one human V gene segment. The human V gene segment is placed downstream of the non-human V gene segments. Even more preferably, the humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another embodiment of the present invention is directed to methods of making a transgenic vector containing a humanized Ig locus by isolating an Ig locus or a portion of an Ig locus from a non-human animal, and integrating the desired human Ig gene segment(s) into the isolated animal Ig locus or the isolated portion of an Ig locus. The human Ig gene segment(s) are integrated into the isolated animal Ig locus or the isolated portion of an Ig locus by ligation or homologous recombination in such a way as to retain the capacity of the locus for undergoing effective gene rearrangement and gene conversion in the non-human animal. Integration of a human Ig gene segment by homologous recombination can be accomplished by using the recombination vectors of the present invention.

In another embodiment, the present invention provides methods of making transgenic animals capable of producing humanized antibodies. The transgenic animals can be made by introducing a transgenic vector containing a humanized Ig locus, or a recombination vector containing a human Ig gene segment, into a recipient cell or cells of an animal, and deriving an animal from the genetically modified recipient cell or cells.

Transgenic animals containing one or more humanized Ig loci, and cells derived from such transgenic animals (such as B cells from an immunized transgenic animal) are also provided. The transgenic animals of the present invention are capable of gene rearranging and gene converting the transgenic humanized Ig loci to produce a diversified repertoire of humanized immunoglobulin molecules.

Brief Description of the Drawings

Figure 1. Cow Cγ 3' flanking sequences. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M1. The sequences of clone 11, clone 3,

and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

Figure 2. Sheep Cγ 3' flanking sequences. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M2. The sequences of clone 11 and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

Figure 3. A novel 3' flanking sequence (SEQ ID NO: 10) of the rabbit Cgamma gene.

Figure 4. A novel nucleotide sequence (SEQ ID NO: 11) 3' of the rabbit Ckappa 1 gene.

Figure 5. Novel nucleotide sequences (SEQ ID NO: 12 and SEQ ID NO: 13) 5' of the rabbit Cgamma gene. The sequences between SEQ ID NO: 12 and SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the M1 and M2 regions 3' of the Cgamma gene.

Figure 7a. DNA construct for the replacement of rabbit Cκ with human Cκ. A 0.5 kb fragment containing a DNA sequence encoding human Ck is flanked by sequences from the rabbit Cκ1 gene. The upstream sequence (5'Cκ) is 2.8 kb, the downstream sequence (3'Cκ) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk casette for negative selection.

Figure 7b. DNA construct for the replacement of rabbit Cγ with human Cγ1. A 1.8 kb fragment containing a DNA sequence encoding human Cγ1 is flanked by sequences from the rabbit Cγ gene. The upstream sequence (5'Cγ) is 1.9 kb, the downstream sequence (3'Cγ) is 3.1 kb. The vector also contains a lox-neo casette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to scale.

Figure 8. DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cγ1 gene segment flanked by 50 nucleotides derived from the flanking regions of rabbit Cγ gene. Flanking sequences derived from the flanking regions of rabbit Cγ gene are underlined.

Figure 9. DNA fragment (SEQ ID NO: 52) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit VH1 and J genes are underlined.

Figure 10. DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin heavy chain Cκ gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappa1 gene. Flanking sequences derived from the flanking regions of rabbit Cκ gene are underlined.

Figure 11. DNA fragment (SEQ ID NO: 54) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit immunoglobulin V and J genes are underlined.

Figure 12. DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides (underlined) derived from the flanking sequences of chicken Clambda gene.

Figure 13. Modification of the chicken light chain locus using the ET system. A chicken genomic BAC clone with the full-length light chain locus was modified by homologous recombination. In a first step Cλ was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human Cλ gene.

Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived from the flanking regions of chicken immunolgobulin V and J genes are underlined.

Figure 15. Modified chicken light chain locus.

Detailed Description of the Invention

One embodiment of the present invention provides humanized immunoglobulins (antibodies).

By "a humanized antibody" or "a humanized immunoglobulin" is meant an immunoglobulin molecule having at least a portion of a human immunoglobulin polypeptide sequence (or a polypeptide sequence encoded by a human Ig gene segment). The humanized immunoglobulin molecules of the present invention can be isolated from a transgenic non-human animal engineered to produce humanized immunoglobulin molecules. Such humanized immunoglobulin molecules are less immunogenic to primates, especially humans, relative to non-humanized immunoglobulin molecules prepared from the animal or prepared from cells derived from the animal.

The term "non-human animals" as used herein includes, but is not limited to, rabbits, pigs, birds (e.g., chickens, turkeys, ducks, geese and the like), sheep, goats, cows and horses. Preferred non-human animals are those animals which rely primarily on gene conversion and/or somatic hypermutation to generate antibody diversity, e.g., rabbit, pigs, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow. Particularly preferred non-human animals are rabbit and chicken.

In animals such as human and mouse, there are multiple copies of V, D and J gene segments on the heavy chain locus, and multiple copies of V and J gene segments on a light chain locus. Antibody diversity in these animals is generated primarily by gene rearrangement, i.e., different combinations of gene segments to form rearranged heavy chain variable region and light chain variable region. In other animals (e.g., rabbit, chicken, sheep, goat, and cow), however, gene rearrangement does not play a significant role in the generation of antibody diversity. For example, in rabbit, only a very limited number of the V gene segments, most often the V gene segments at the 3' end of the V-region, are used in gene rearrangement to form a contiguous VDJ segment. In chicken, only one V gene segment (the one adjacent to the D region, or "the 3' proximal V gene segment"), one D segment and one J segment are used in the heavy chain rearrangement; and only one V gene segment (the 3' proximal V segment) and one J segment are used in the light chain rearrangement. Thus, in these animals, there is little diversity among initially rearranged variable region sequences resulting from junctional diversification. Further diversification of the rearranged Ig genes is achieved by gene conversion, a

process in which short sequences derived from the upstream V gene segments replace short sequences within the V gene segment in the rearranged Ig gene.

The term "Ig gene segment" as used herein refers to segments of DNA encoding various portions of an Ig molecule, which are present in the germline of animals and humans, and which are brought together in B cells to form rearranged Ig genes. Thus, Ig gene segments as used herein include V gene segments, D gene segments, J gene segments and C region gene segments.

The term "human Ig gene segment" as used herein includes both naturally occurring sequences of a human Ig gene segment, degenerate forms of naturally occurring sequences of a human Ig gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially identical to the polypeptide encoded by a naturally occurring sequence of a human Ig gene segment. By "substantially" is meant that the degree of amino acid sequence identity is at least about 85%-95%.

A preferred humanized immunoglobulin molecule of the present invention contains at least a portion of a human heavy or light chain constant region polypeptide sequence. A more preferred immunoglobulin molecule contains at least a portion of a human heavy or light chain constant region polypeptide sequence, and at least a portion of a human variable domain polypeptide sequence.

In another embodiment of the present invention, a preparation of humanized antibodies is provided.

By "a preparation of humanized antibodies" or "a humanized antibody preparation" is meant an isolated antibody product or a purified antibody product prepared from a transgenic non-human animal (e.g., serum, milk, or egg yolk of the animal) or from cells derived from a transgenic non-human animal (e.g., a B-cell or a hybridoma cell).

A humanized antibody preparation can be a preparation of polyclonal antibodies, which includes a repertoire of humanized immunoglobulin molecules. A humanized antibody preparation can also be a preparation of a monoclonal antibody.

Although the immunogenicity to humans of a humanized monoclonal antibody preparation is also reduced as compared to a non-humanized monoclonal antibody preparation, humanized polyclonal antibody preparations are preferred embodiments of

the present invention. It has been recognized that humanized monoclonal antibodies still invoke some degree of an immune response (an anti-idiotype response) in primates (e.g., humans) when administered repeatedly in large quantities because of the unique and novel idiotype of the monoclonal antibody. The present inventors have uniquely recognized that the overall immunogenicity of polyclonal antibodies is less dependent on an anti-idiotype response. For example, polyclonal antibodies made from non-human animals with only the constant region elements humanized (e.g., polyclonal antibodies having constant regions encoded by human gene segments, and having variable domains encoded by the endogenous genes of the non-human animal), are substantially non-immunogenic to primates.

Without intending to be bound to any theory, the present inventors have proposed that the reduced immunogenicity of such a humanized polyclonal antibody preparation is due to the fact that the preparation contains a very large number of different antibodies with many different idiotypes which are to a large extent defined by novel amino acid sequences in the complimentarity determining regions (CDR) of the heavy and light chain. Therefore, upon administration of such preparation into a primate such as a human, the administered amount of each individual immunoglobulin molecule in the preparation may be too low to solicit immune response against each immunoglobulin molecule. Thus, the humanized polyclonal antibody preparation which has many different idiotypes and variable regions has minimal immunogenicity to a recipient, even if the antibodies in the polyclonal antibody preparation are all directed to the same antigen. To further reduce any potential residual immunogenicity, a humanized polyclonal antibody preparation may be prepared which is composed of immunoglobulin molecules having both the variable domains and the constant regions encoded by human Ig gene segments.

In a preferred embodiment, the present invention provides an antibody preparation which includes humanized immunoglobulin molecules having at least a portion of a human heavy or light chain constant region polypeptide sequence. More preferably, the humanized immunoglobulines in the antibody preparation of the present invention further contain at least a portion of a human variable domain polypeptide

sequence, in addition to at least a portion of a human constant region polypeptide sequence.

Preferred humanized antibody preparations of the present invention are composed of humanized antibodies made from transgenic non-human animals whose antibody diversity is generated primarily by gene conversion, such as rabbit, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow; preferably, rabbit and chicken.

Once a transgenic non-human animal capable of producing diversified humanized immunoglobulin molecules is made (as further set forth below), humanized immunoglobulins and humanized antibody preparations against an antigen can be readily obtained by immunizing the animal with the antigen. A variety of antigens can be used to immunize a transgenic host animal. Such antigens include, microorganism, e.g. viruses and unicellular organisms (such as bacteria and fungi), alive, attenuated or dead, fragments of the microorganisms, or antigenic molecules isolated from the microorganisms.

Preferred bacterial antigens for use in immunizing an animal include purified antigens from *Staphylococcus aureus* such as capsular polysaccharides type 5 and 8, recombinant versions of virulence factors such as alpha-toxin, adhesin binding proteins, collagen binding proteins, and fibronectin binding proteins. Preferred bacterial antigens also include an attenuated version of *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, and *Klebsiella pneumoniae*, or culture supernatant from these bacteria cells. Other bacterial antigens which can be used in immunization include purified lipopolysaccharide (LPS), capsular antigens, capsular polysaccharides and/or recombinant versions of the outer membrane proteins, fibronectin binding proteins, endotoxin, and exotoxin from Pseudomonas aeruginosa, enterococcus, enterobacter, and Klebsiella pneumoniae.

Preferred antigens for the generation of antibodies against fungi include attenuated version of fungi or outer membrane proteins thereof, which fungi include, but are not limited to, Candida albicans, Candida parapsilosis, Candida tropicalis, and Cryptococcus neoformans.

Preferred antigens for use in immunization in order to generate antibodies against viruses include the envelop proteins and attenuated versions of viruses which include, but are not limited to respiratory synctial virus (RSV) (particularly the F-Protein), Hepatitis C virus (HCV), Hepatits B virus (HBV), cytomegalovirus (CMV), EBV, and HSV.

Therapeutic antibodies can be generated for the treatment of cancer by immunizing transgenic animals with isolated tumor cells or tumor cell lines; tumor-associated antigens which include, but are not limited to, Her-2-neu antigen (antibodies against which are useful for the treatment of breast cancer); CD20, CD22 and CD53 antigens (antibodies against which are useful for the treatment of B cell lymphomas), (3) prostate specific membrane antigen (PMSA) (antibodies against which are useful for the treatment of prostate cancer), and 17-1A molecule (antibodies against which are useful for the treatment of colon cancer).

The antigens can be administered to a transgenic host animal in any convenient manner, with or without an adjuvant, and can be administered in accordance with a predetermined schedule.

After immunization, serum or milk from the immunized transgenic animals can be fractionated for the purification of pharmaceutical grade polyclonal antibodies specific for the antigen. In the case of transgenic birds, antibodies can also be made by fractionating egg yolks. A concentrated, purified immunoglobulin fraction may be obtained by chromatography (affinity, ionic exchange, gel filtration, etc.), selective precipitation with salts such as ammonium sulfate, organic solvents such as ethanol, or polymers such as polyethyleneglycol.

For making a monoclonal antibody, spleen cells are isolated from the immunized transgenic animal and used either in cell fusion with transformed cell lines for the production of hybridomas, or cDNAs encoding antibodies are cloned by standard molecular biology techniques and expressed in transfected cells. The procedures for making monoclonal antibodies are well established in the art. See, e.g., European Patent Application 0 583 980 A1 ("Method For Generating Monoclonal Antibodies From Rabbits"), U.S. Patent No. 4,977,081 ("Stable Rabbit-Mouse Hybridomas And Secretion

Products Thereof"), WO 97/16537 ("Stable Chicken B-cell Line And Method of Use Thereof"), and EP 0 491 057 B1 ("Hybridoma Which Produces Avian Specific Immunoglobulin G"), the disclosures of which are incorporated herein by reference. In vitro production of monoclonal antibodies from cloned cDNA molecules has been described by Andris-Widhopf et al., "Methods for the generation of chicken monoclonal antibody fragments by phage display", *J Immunol Methods 242:159* (2000), and by Burton, D. R., "Phage display", *Immunotechnology 1:87* (1995), the disclosures of which are incorporated herein by reference.

In a further embodiment of the present invention, purified monoclonal or polyclonal antibodies are admixed with an appropriate pharmaceutical carrier suitable for administration in primates especially humans, to provide pharmaceutical compositions. Pharmaceutically acceptable carriers which can be employed in the present pharmaceutical compositions can be any and all solvents, dispersion media, isotonic agents and the like. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of the antibodies contained therein, its use in the pharmaceutical compositions of the present invention is appropriate. The carrier can be liquid, semi-solid, e.g. pastes, or solid carriers. Examples of carriers include oils, water, saline solutions, alcohol, sugar, gel, lipids, liposomes, resins, porous matrices, binders, fillers, coatings, preservatives and the like, or combinations thereof

The present invention is further directed to novel nucleotide sequences and vectors, as well as the use of the sequences and vectors in making a transgenic non-human animal which produces humanized immunoglobulins.

In general, the genetic engineering of a non-human animal involves the integration of one or more human Ig gene segments into the animal's genome to create one or more humanized Ig loci. It should be recognized that, depending upon the approach used in the genetic modification, a human Ig gene segment can be integrated at the endogenous Ig locus of the animal (as a result of targeted insertion, for example), or at a different locus of the animal. In other words, a humanized Ig locus can reside at the chromosomal location where the endogenous Ig locus of the animal ordinarily resides, or at a chromosomal location other than where the endogenous Ig locus of the animal

ordinarily resides. Regardless of the chromosomal location, a humanized Ig locus of the present invention has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulin molecules. An Ig locus having the capacity to undergo gene rearrangement and gene conversion is also referred to herein as a "functional" Ig locus, and the antibodies with a diversity generated by a functional Ig locus are also referred to herein as "functional" antibodies or a "functional" repertoire of antibodies.

In one embodiment, the present invention provides novel sequences useful for creating a humanized Ig locus and making transgenic animals capable of producing humanized immunoglobulin molecules. In particular, the present invention provides sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating antibody diversity (e.g., rabbit, pigs, sheep, goat, cow, birds such as chicken, turkey, duck, goose, and the like).

The 5' and 3' flanking regions of the genes coding for the constant region are particularly important as these sequences contain untranslated regulatory elements (e.g., enhancers) critical for high Ig expression in the serum. The 3' flanking region of the genes coding for the constant region of the heavy chain also contain exons coding for the membranous and cytoplasmic tail of the membrane form of immunoglobulin (Volgina et al. *J Immunol* 165:6400, 2000). It has been previously established that the membrane and cytoplasmic tail of the membrane form of antibodies are critical in achieving a high level of expression of the antibodies in mice sera (Zou et al., *Science* 262:1271, 1993). Thus, the identification of the flanking sequences permits the replacement of exons and intervening introns of the Cγ gene with the human equivalent, and the maintenance of the endogenous exons encoding the transmembrane and cytoplasmic tail regions as well as the endogenous non-coding enhancer sequences.

In one embodiment, the present invention provides 3' flanking sequences of heavy chain constant regions of non-human animals. More particularly, nucleotide sequences downstream (3', 3-prime) of the genes coding for rabbit Cγ, cow Cγ1,2,3, and sheep Cγ1,2 are provided. Especially preferred nucleotide sequences include SEQ ID NO:

10 (3' of rabbit Cγ), SEQ ID NOS: 3-5 (3' of cow Cγ1,2,3), and SEQ ID NOS: 8-9 (3' of sheep Cγ1,2).

In another embodiment, the present invention provides 3' flanking sequences of light chain constant regions of non-human animals. More particularly, the present invention provides nucleotide sequences downstream (3', 3-prime) of the genes coding for Cκ in rabbits. Especially preferred nucleotide sequences include SEQ ID NO: 11 (3' of rabbit Cκ).

In still another embodiment, the present invention provides 5' flanking sequences of heavy chain constant regions of non-human animals. More particularly, nucleotide sequences upstream (5', 5-prime) of the rabbit Cγ gene are provided. Especially preferred sequences include SEQ ID NO: 12 and SEQ ID NO: 13.

Another embodiment of the present invention provides 5' flanking sequences of light chain constant regions of non-human animals.

Portions of the above novel flanking sequences are provided by the present invention. By "a portion" is meant a fragment of a flanking nucleotide sequence capable of mediating homologous recombination between the human Ig gene segment and the target animal Ig gene segment. Generally, a portion is at least about 200 base pairs, preferably, at least about 400 base pairs, for recombination in animal cells such as ES cells or fibroblasts, and at least about 40 base pairs, preferably at least about 50 base pairs, for recombination in *E. coli*. Examples of portions of the above novel flanking sequences include SEQ ID NOS: 59-60, 61-62, 63-64, 65-66, 67-68 and 69-70 (represented by the underlined sequences in Figures 8-12 and 14, respectively).

In a further aspect, the present invention provides vectors useful for the replacement of an Ig gene segment of a non-human animal with the corresponding human Ig gene segment. These vectors, also referred to herein as "recombination vectors", include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences have a degree of homology with the flanking sequences of the target animal Ig gene segment sufficient to mediate homologous recombination between the human gene and the animal gene segments. Generally, at least about 200 bases should be identical between the flanking regions in a recombination

vector and the flanking regions of the target gene to achieve efficient homologous recombination in animal cells such as ES cells and fibroblasts; and at least about 40 bases should be identical to achieve efficient homologous recombination in *E. coli*.

Recombination vectors useful for replacing the animal's immunoglobulin heavy chain constant region genes are provided, which contain from 5' to 3', a nucleotide sequence homologous to the 5' flanking region of the target animal heavy chain constant region gene, a human heavy chain constant region gene (e.g., human Cγ1), and a nucleotide sequence homologous to the 3' flanking region of the target animal heavy chain constant region gene.

Preferred recombination vectors are provided for the replacement of the rabbit heavy chain constant region genes. One such vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13 or a portion thereof, a human heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of or SEQ ID NO: 10. Another such vector contains SEQ ID NO: 51 (Figure 8) which is characterized as having a human Cγ1 gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

Recombination vectors are also provided which are useful for replacing the animal's immunoglobulin light chain constant region genes. Such vectors contain from 5' to 3', a nucleotide sequence homologous to the 5' flanking region of the target light chain constant region gene, a human light chain constant region gene (e.g., human Cκ or Cλ), and a nucleotide sequence homologous to the 3' flanking region of the target light chain constant region gene.

Preferred vectors include those useful for replacing the rabbit light chain constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human Cκ linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain Cκ1 gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A

recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

The recombination vectors of the present invention can include additional sequences that facilitate the selection of cells which have undergone a successful recombination event. For example, marker genes coding for resistance to neomycin, bleomycin, puromycin and the like can be included in the recombination vectors to facilitate the selection of cells which have undergone a successful recombination event.

In a further aspect of the present invention, transgenic constructs or vectors carrying one or more humanized Ig loci are provided.

In one embodiment, the present invention provides transgenic constructs containing a humanized Ig heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in such humanized heavy chain locus are juxtaposed wit respect to each other in an unrearranged configuration (or "the germline configuration"), or in a partially or fully rearranged configuration. The humanized heavy chain locus has the capacity to undergo gene rearrangement (if the gene segments are not fully rearranged) and gene conversion in the non-human animal thereby producing a diversified repertoire of heavy chains having human polypeptide sequences, or "humanized heavy chains".

In a preferred embodiment, the humanized heavy chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, Cα or Cγ (including any of the Cγ subclasses 1, 2, 3 and 4).

In another more preferred embodiment, the humanized heavy chain locus of the transgene contains a humanized V-region and a humanized C-region, i.e., a V-region having at least one human VH gene segment and a C-region having at least one human C gene segment (e.g., human Cα or Cγ).

Preferably, the humanized V-region includes at least about 10-100 heavy chain V (or "VH") gene segments, at least one of which is a human VH gene segment. In accordance with the present invention, the human VH gene segment included in the

transgene shares at least about 75% to about 85% homology to the VH gene segments of the host animal, particularly those animal VH gene segments included in the upstream region of the transgene. As described above, a human VH segment encompasses naturally occurring sequences of a human VH gene segment, degenerate forms of naturally occurring sequences of a human VH gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human heavy chain V domain polypeptide.

Preferably, the human VH gene segment(s) is placed downstream of the non-human VH segments in the transgene locus. Preferably, the non-human VH gene segments in the transgene are the VH gene segments from the 3' VH-region in the Ig locus of the host animal, including the 3' proximal VH1.

In another embodiment, the present invention provides transgenic constructs containing a humanized light chain locus capable of undergoing gene rearrangement and gene conversion in the host animal thereby producing a diversified repertoire of light chains having human polypeptide sequences, or "humanized light chains".

The humanized light locus includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed in an unrearranged configuration (or "the germline configuration"), or fully rearranged configuration.

In a preferred embodiment, the humanized light chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, Cλ or Cκ.

In another preferred embodiment, the humanized light chain locus of the transgene contains a humanized V-region and a humanized C-region, e.g., a V-region having at least one human VL gene and/or at least one rearranged human VJ segment, and a C-region having at least one human C gene segment (e.g., human Cλ or Cκ).

Preferably, the humanized V-region includes at least about 10-100 light chain V (or "VL") gene segments, at least one of which is a human VL gene segment. The human VL gene segment included in the transgene shares at least about 75% to about 85%

homology to the VL gene segments of the host animal, particularly those animal VL gene segments included in the upstream region of the transgene. Consistently, a human VL segment encompasses naturally occurring sequences of a human VL gene segment, degenerate forms of naturally occurring sequences of a human VL gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human light chain V domain polypeptide.

Preferably, the human VL gene segment(s) is placed downstream of the non-human VL segments in the transgene locus. The non-human VL gene segments in the transgene construct are selected from the VL gene segments in the 3'VL-region in the light chain locus of the host animal, including the 3' proximal VL1.

In still another preferred embodiment, the humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another aspect of the present invention is directed to methods of making a transgenic vector containing a humanized Ig locus. Such methods involve isolating an Ig locus or a portion thereof from a non-human animal, and inserting the desired human Ig gene segment(s) into the isolated animal Ig locus or the isolated portion of an animal Ig locus. The human Ig gene segment(s) are inserted into the isolated animal Ig locus or a portion thereof by ligation or homologous recombination in such a way as to retain the capacity of the locus of undergoing effective gene rearrangement and gene conversion in the non-human animal.

Preferably, DNA fragments containing an Ig locus to be humanized are isolated from animals which generate antibody diversity by gene conversion, e.g., rabbit and chicken. Such large DNA fragments can be isolated by screening a library of plasmids, cosmids, YACs or BACs, and the like, prepared from the genomic DNA of the non-human animal. An entire animal C-region can be contained in one plasmid or cosmid clone which is subsequently subjected to humanization. YAC clones can carry DNA fragments of up to 2 megabases, thus an entire animal heavy chain locus or a large portion thereof can be isolated in one YAC clone, or reconstructed to be contained in one YAC clone. BAC clones are capable of carrying DNA fragments of smaller sizes (about 150-

250 kb). However, multiple BAC clones containing overlapping fragments of an Ig locus can be separately humanized and subsequently injected together into an animal recipient cell, wherein the overlapping fragments recombine in the recipient animal cell to generate a continuous Ig locus.

Human Ig gene segments can be integrated into the Ig locus on a vector (e.g., a BAC clone) by a variety of methods, including ligation of DNA fragments, or insertion of DNA fragments by homologous recombination. Integration of the human Ig gene segments is done in such a way that the human Ig gene segment is operably linked to the host animal sequence in the transgene to produce a functional humanized Ig locus, i.e., an Ig locus capable of gene rearrangement and gene conversion which lead to the production of a diversified repertoire of humanized antibodies.

Preferably, human Ig gene segments are integrated into the Ig locus by homologous recombination. Homologous recombination can be performed in bacteria, yeast and other cells with a high frequency of homologous recombination events. For example, a yeast cell is transformed with a YAC containing an animal's Ig locus or a large portion thereof. Subsequently, such yeast cell is further transformed with a recombination vector as described hereinabove, which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' flanking sequence. The 5' and the 3' flanking sequences in the recombination vector are homologous to those flanking sequences of the animal Ig gene segment on the YAC. As a result of a homologous recombination, the animal Ig gene segment on the YAC is replaced with the human Ig gene segment. Alternatively, a bacterial cell such as *E. coli* is transformed with a BAC containing an animal's Ig locus or a large portion thereof. Such bacterial cell is further transformed with a recombination vector which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' flanking sequence. The 5' and the 3' flanking sequences in the recombination vector mediate homologous recombination and exchange between the human Ig gene segment on the recombination vector and the animal Ig gene segment on the BAC. Humanized YACs and BACs can be readily isolated from the cells and used in making transgenic animals.

In a further aspect of the present invention, methods of making transgenic animals capable of producing humanized immunoglobulins are provided.

According to the present invention, a transgenic animal capable of making humanized immunoglobulins are made by introducing into a recipient cell or cells of an animal one or more of the transgenic vectors described herein above which carry a humanized Ig locus, and deriving an animal from the genetically modified recipient cell or cells.

Preferably, the recipient cells are from non-human animals which generate antibody diversity by gene conversion and hypermutation, e.g., bird (such as chicken), rabbit, cows and the like. In such animals, the 3'proximal V gene segment is preferentially used for the production of immunoglobulins. Integration of a human V gene segment into the Ig locus on the transgene vector, either by replacing the 3'proximal V gene segment of the animal or by being placed in close proximity of the 3'proximal V gene segment, results in expression of human V region polypeptide sequences in the majority of immunoglobulins. Alternatively, a rearranged human V(D)J segment may be inserted into the J locus of the immunoglobulin locus on the transgene vector.

The transgenic vectors containing a humanized Ig locus is introduced into the recipient cell or cells and then integrated into the genome of the recipient cell or cells by random integration or by targeted integration.

For random integration, a transgenic vector containing a humanized Ig locus can be introduced into an animal recipient cell by standard transgenic technology. For example, a transgenic vector can be directly injected into the pronucleus of a fertilized oocyte. A transgenic vector can also be introduced by co-incubation of sperm with the transgenic vector before fertilization of the oocyte. Transgenic animals can be developed from fertilized oocytes. Another way to introduce a transgenic vector is by transfecting embryonic stem cells and subsequently injecting the genetically modified embryonic stem cells into developing embryos. Alternatively, a transgenic vector (naked or in combination with facilitating reagents) can be directly injected into a developing embryo. Ultimately, chimeric transgenic animals are produced from the embryos which contain the humanized Ig transgene integrated in the genome of at least some somatic cells of the transgenic animal.

In a preferred embodiment, a transgene containing a humanized Ig locus is randomly integrated into the genome of recipient cells (such as fertilized oocyte or developing embryos) derived from animal strains with an impaired expression of endogenous immunoglobulin genes. The use of such animal strains permits preferential expression of immunoglobulin molecules from the humanized transgenic Ig locus. Examples for such animals include the Alicia and Basilea rabbit strains, as well as Agammaglobinemic chicken strain. Alternatively, transgenic animals with humanized immunoglobulin transgenes or loci can be mated with animal strains with impaired expression of endogenous immunoglobulins. Offspring homozygous for an impaired endogenous Ig locus and a humanized transgenic Ig locus can be obtained.

For targeted integration, a transgenic vector can be introduced into appropriate animal recipient cells such as embryonic stem cells or already differentiated somatic cells. Afterwards, cells in which the transgene has integrated into the animal genome and has replaced the corresponding endogenous Ig locus by homologous recombination can be selected by standard methods. The selected cells may then be fused with enucleated nuclear transfer unit cells, e.g. oocytes or embryonic stem cells, cells which are totipotent and capable of forming a functional neonate. Fusion is performed in accordance with conventional techniques which are well established. See, for example, Cibelli et al., Science (1998) 280:1256. Enucleation of oocytes and nuclear transfer can also be performed by microsurgery using injection pipettes. (See, for example, Wakayama et al., Nature (1998) 394:369.) The resulting egg cells are then cultivated in an appropriate medium, and transferred into synchronized recipients for generating transgenic animals. Alternatively, the selected genetically modified cells can be injected into developing embryos which are subsequently developed into chimeric animals.

Further to the present invention, a transgenic animal capable of producing humanized immunoglobulins can also be made by introducing into a recipient cell or cells, one or more of the recombination vectors described herein above, which carry a human Ig gene segment, linked to 5' and 3' flanking sequences that are homologous to the flanking sequences of the endogenous Ig gene segment, selecting cells in which the endogenous Ig

gene segment is replaced by the human Ig gene segment by homologous recombination, and deriving an animal from the selected genetically modified recipient cell or cells.

Similar to the target insertion of a transgenic vector, cells appropriate for use as recipient cells in this approach include embryonic stem cells or already differentiated somatic cells. A recombination vector carrying a human Ig gene segment can be introduced into such recipient cells by any feasible means, e.g., transfection. Afterwards, cells in which the human Ig gene segment has replaced the corresponding endogenous Ig gene segment by homologous recombination, can be selected by standard methods. These genetically modified cells can serve as nuclei donor cells in a nuclear transfer procedure for cloning a transgenic animal. Alternatively, the selected genetically modified embryonic stem cells can be injected into developing embryos which can be subsequently developed into chimeric animals.

Transgenic animals produced by any of the foregoing methods form another embodiment of the present invention. The transgenic animals have at least one, i.e., one or more, humanized Ig loci in the genome, from which a functional repertoire of humanized antibodies is produced.

In a preferred embodiment, the present invention provides transgenic rabbits having one or more humanized Ig loci in the genome. The transgenic rabbits of the present invention are capable of rearranging and gene converting the humanized Ig loci, and expressing a functional repertoire of humanized antibodies.

In another preferred embodiment, the present invention provides transgenic chickens having one or more humanized Ig loci in the genome. The transgenic chickens of the present invention are capable of rearranging and gene converting the humanized Ig loci, and expressing a functional repertoire of humanized antibodies.

Cells derived from the transgenic animals of the present invention, such as B cells or cell lines established from a transgenic animal immunized against an antigen, are also part of the present invention.

In a further aspect of the present invention, methods are provided for treating a disease in a primate, in particular, a human subject, by administering a purified humanized

antibody composition, preferably, a humanized polyclonal antibody composition, desirable for treating such disease.

The humanized polyclonal antibody compositions used for administration are generally characterized by containing a polyclonal antibody population, having immunoglobulin concentrations from 0.1 to 100 mg/ml, more usually from 1 to 10 mg/ml. The antibody composition may contain immunoglobulins of various isotypes. Alternatively, the antibody composition may contain antibodies of only one isotype, or a number of selected isotypes.

In most instances the antibody composition consists of unmodified immunoglobulins, i.e., humanized antibodies prepared from the animal without additional modification, e.g., by chemicals or enzymes. Alternatively, the immunoglobulin fraction may be subject to treatment such as enzymatic digestion (e.g. with pepsin, papain, plasmin, glycosidases, nucleases, etc.), heating, etc, and/or further fractionated.

The antibody compositions generally are administered into the vascular system, conveniently intravenously by injection or infusion via a catheter implanted into an appropriate vein. The antibody composition is administered at an appropriate rate, generally ranging from about 10 minutes to about 24 hours, more commonly from about 30 minutes to about 6 hours, in accordance with the rate at which the liquid can be accepted by the patient. Administration of the effective dosage may occur in a single infusion or in a series of infusions. Repeated infusions may be administered once a day, once a week once a month, or once every three months, depending on the half-life of the antibody preparation and the clinical indication. For applications on epithelial surfaces the antibody compositions are applied to the surface in need of treatment in an amount sufficient to provide the intended end result, and can be repeated as needed.

The antibody compositions can be used to bind and neutralize antigenic entities in human body tissues that cause disease or that elicit undesired or abnormal immune responses. An "antigenic entity" is herein defined to encompass any soluble or cell-surface bound molecules including proteins, as well as cells or infectious disease-causing organisms or agents that are at least capable of binding to an antibody and preferably are also capable of stimulating an immune response.

Administration of an antibody composition against an infectious agent as a monotherapy or in combination with chemotherapy results in elimination of infectious particles. A single administration of antibodies decreases the number of infectious particles generally 10 to 100 fold, more commonly more than 1000-fold. Similarly, antibody therapy in patients with a malignant disease employed as a monotherapy or in combination with chemotherapy reduces the number of malignant cells generally 10 to 100 fold, or more than 1000-fold. Therapy may be repeated over an extended amount of time to assure the complete elimination of infectious particles, malignant cells, etc. In some instances, therapy with antibody preparations will be continued for extended periods of time in the absence of detectable amounts of infectious particles or undesirable cells. Similarly, the use of antibody therapy for the modulation of immune responses may consist of single or multiple administrations of therapeutic antibodies. Therapy may be continued for extended periods of time in the absence of any disease symptoms.

The subject treatment may be employed in conjunction with chemotherapy at dosages sufficient to inhibit infectious disease or malignancies. In autoimmune disease patients or transplant recipients, antibody therapy may be employed in conjunction with immunosuppressive therapy at dosages sufficient to inhibit immune reactions.

The invention is further illustrated, but by no means limited, by the following examples.

Example 1

Novel Sequences 3'prime of the Cγ Gene from Cows, Sheep and Rabbits

Genomic DNA was isolated from blood of a Simmental cow using the QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the cow Cγ gene (i.e., the cow Cγ gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA as template and the following primers:

5' primer: 5'cgcaagcttCCTACACGTGTGTGGTGATG3' (SEQ ID NO: 1);

3' primer: 5'cgcaagcttAAGATGGWGATGGTSGTCCA3' (SEQ ID NO: 2)(Universal degenerate code: W=(A/T) S=(G/C)).

The upper-case portion of the 5' primer was from exon 3 of Cγ, and the lower-case portion represented a terminal HindIII restriction site. The upper-case portion of the 3' primer was a degenerate sequence designed according to the published sequences from the human M1 exon and the mouse M1 exon, and the lower-case portion represented a terminal HindIII restriction site. A 1.3kb PCR fragment was obtained using the EXPAND long template PCR system (Roche). The fragment was gel purified, digested with HindIII, and cloned into a Bluescript cloning vector. The resulting clones fell into three populations, which differ from one another in the pattern of the restriction fragments obtained with BamHI, EcoRI and XhoI. One clone from each population was sequenced, and the sequences are shown in Figure 1 (SEQ ID NOS: 3-5).

Genomic DNA was isolated from blood of a Merino sheep using the QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the sheep Cγ gene (i.e., the sheep Cγ gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA as template and the following primers:

5' primer: 5'cgcggatccCCTACGCGTGTGTGGTGATG3' (SEQ ID NO: 6)

3' primer: 5'cgcggatccACCGAGGAGAAGATCCACTT3' (SEQ ID NO: 7)

The upper-case portion of the 5' primer was from exon 3 of Cγ, and the lower-case portion represented a terminal BamHI restriction site. The upper-case portion of the 3' primer was designed according to the published sequences from the human M2 exon and the mouse M2 exon, and the lower-case portion represented a terminal BamHI restriction site. A 2.9kb PCR fragment was obtained using the EXPAND long template PCR system (Roche). The fragment was gel purified, digested with BamHI, and cloned into a Bluescript cloning vector. The resulting clones fell into two populations, which differ from each other in the pattern of the restriction fragments obtained with HindIII, EcoRI and XhoI. One clone from each population was sequenced, and the sequences are shown in Figure 2 (SEQ ID NOS: 8-9).

A 10kb EcoRI fragment containing the Cγ gene and its flanking sequences from A2 allotype rabbit was subcloned from a genomic cosmid clone (cos 8.3 from

Knight et al., *J Immunol* (1985) 1245-50, "Organization and polymorphism of rabbit immunoglobulin heavy chain genes"). The nucleotide sequences 5' and 3' of Cγ were determined using standard methods and are set forth in Figure 3 and 5, SEQ ID NO: 10, 12, 13, respectively.

Sequences 3' of rabbit Ckappa1 were determined from an EcoRI/BamHI subclone from VJk2Ck In pSV2neo. The nucleotide sequence is set forth in Figure 4, SEQ ID NO: 11.

The amino acid sequences encoded by the M1 and M2 exons from cow, sheep and rabbit were deduced from the above 3' flanking sequence. These amino acid sequences were aligned with the published M1 and M2 sequences from camel, human and mouse, as shown in Figure 6.

Example 2

A Vector for Replacing the Rabbit Endogenous Cγ Gene Segment with the Human Cγ1 Segment

Genomic DNA is isolated from rabbit fetal fibroblasts of an a2-homozygous rabbit. The DNA sequence upstream of rabbit Cγ (i.e., the 5' flanking sequence of rabbit Cγ) is amplified by PCR using the following primers:

5' taattatgcggccgcCTTCAGCGTGAACCACGCCCTC 3' (SEQ ID NO: 39) with a 5' NotI site and

5' GTCGACGCCCCTCGATGCACTCCCAGAG 3' (SEQ ID NO: 40).

The DNA sequence downstream of rabbit Cγ (i.e., the 3' flanking sequence of rabbit Cγ) is amplified with the following primers:

5' ggtaccCTCTCCCTCCCCCACGCCGCAGC 3' (SEQ ID NO: 41) with a 5' KpnI site and

5' atatctcagaACTGGCTGTCCCTGCTGTAGTACACGG 3' (SEQ ID NO: 42) with a 5' XhoI site.

Human genomic DNA is isolated from human peripheral blood lymphocytes. The DNA fragment encoding human Cγ1 is amplified using the following primers:

5' GTCGACACTGGACGCTGAACCTCGCGG 3' (SEQ ID NO: 43) and

5′ GGTACCGGGGGCTTGCCGGCCGTCGCAC 3′ (SEQ ID NO: 44).

The fragments are digested with restriction enzymes and cloned into a Bluescript vector. Subsequently, a lox neo-cassette is inserted into the SalI site and an Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in Figure 7a.

Example 3

A Vector for Replacing the Rabbit Endogenous Cκ Gene Segment with the Human Cκ Segment

Genomic DNA was isolated from rabbit fetal fibroblasts of a b5-homozygous rabbit. The DNA sequence upstream of rabbit Cκ1 (i.e., the 5' flanking sequence of rabbit Cκ1) was amplified by PCR using the following primers:

5′ gcggccgcTGGCGAGGAGACCAAGCTGGAGATCAAACG 3′ (SEQ ID NO: 45) with a 5′ NotI site

5′ GTCGACGCAGCCCAAAGCTGTTGCAATGGGGCAGCG 3′ (SEQ ID NO: 46).

The DNA sequence downstream of rabbit Cκ1 (i.e., the 5' flanking sequence of rabbit Cκ1) was amplified with the following primers:

5′ atatggtaccGCGAGACGCCTGCCAGGGCACCGCC 3′ (SEQ ID NO: 47) with a 5′ KpnI site

5′ GGATCCCGAGCTTTATGGGCAGGGTGGGGG 3′ (SEQ ID NO: 48).

Human genomic DNA was isolated from human peripheral blood lymphocytes. The DNA fragment encoding human Cκ was amplified using the following primers:

5′ ATATGTCGACCTGGGATAAGCATGCTGTTTTCTGTCTGTCCC 3′ (SEQ ID NO: 49)

5′ CTAGGTACCAGCAGGTGGGGGCACTTCTCCC 3′ (SEQ ID NO: 50).

The fragments were digested with restriction enzymes and cloned into a Bluescript vector. Subsequently, a lox neo-cassette was inserted into the SalI site and an Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in Figure 7b.

Example 4

Replacement of the Endogenous Cγ and Cκ Gene Segments in Rabbit Fetal Fibroblasts with the Corresponding Human Gene Segments

Rabbit fetal fibroblast cells are prepared by standard methods. After one passage, fibroblasts are transfected with 5μg of the NotI-linearized targeting vector as shown in Figure 5a for Cγ or Figure 51b for Cκ, and are seeded in 96-well plates (2×10^3 cells/well). After a positive selection with 600μg/ml G418 and a negative selection with 200nM FIAU, resistant colonies are replica-plated to two 96-well plates for DNA analysis and cryopreservation, respectively. PCR and/or Southern blot analysis is performed to identify cells with the human Cγ1 gene segment integrated in the genome. The cells having the integrated human Cγ1 gene are used in rabbit cloning as described in Example 5.

Example 5

Cloning of Rabbits

Mature Dutch Belton rabbits are superovulated by subcutaneous injection of follicle stimulating hormone (FSH) every 12 hours (0.3 mg x 2 and 0.4 mg x 4). Ovulation is induced by intravenous administration of 0.5 mg luteinizing hormone (LH) 12 hours after the last FSH injection. Oocytes are recovered by ovidual flush 17 hours after LH injection. Oocytes are mechanically enucleated 16-19 hours after maturation. Chromosome removal is assessed with bisBENZIMIDE (HOECHST 33342, Sigma, St. Louis, MO) dye under ultraviolet light. Enucleated oocytes are fused with actively dividing fibroblasts by using one electrical pulse of 180 V/cm for 15 us (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 3-5 hours oocytes are chemically activated with calcium ionophore (6 uM) for 4 min (# 407952, Calbiochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 medium (Specialty Media, Lavalett, NJ) with 3 mg/ml bovine serum albumin (fatty acid free, Sigma) for 3 hours. Following the activation, the embryos are washed in hamster embryo culture

medium (HECM)-Hepes five times and subsequently, cultivated in CR2 medium containing 3 mg/ml fatty-acid free BSA for 2-48 hours at 37.8° C and 5%CO_2 in air. Embryos are then transferred into synchronized recipients. Offsprings are analyzed by PCR for a segment of the transgene.

Example 6

Construction of a DNA Fragment Containing a Portion of a Rabbit Heavy Chain Locus with a Human Cγ1 Gene Segment and a VH Gene Segment Encoding a Human VH Domain Polypeptide Sequence

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit heavy chain Cγ gene from an a2-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 51) is generated by PCR using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit Cγ gene, the human Cγ1 gene, and a sequence derived from the 3' flanking region of the rabbit Cγ gene (Figure 8).

A genomic BAC library derived from an a2-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit Cγ. A BAC clone containing rabbit heavy chain gene segments is identified. The rabbit Cγ gene on this BAC clone is replaced with the human Cγ1 gene by homologous recombination in *E.coli* using the DNA fragment of SEQ ID NO: 51 and the pET system. This replacement is accomplished by two consecutive recombination steps: first the rabbit Cγ gene segment is replaced with a marker gene; then the marker gene is replaced the human Cγ1 gene segment.

The modified BAC clone containing rabbit heavy chain genes and the inserted human Cγ1 gene is further modified by replacing the 3'proximal VH1 segment with a synthetic VH gene segment (Figure 9). This synthetic VH gene segment (SEQ ID NO: 52) is made using overlapping oligonculeotides and includes a 5' flanking sequence, a 3' flanking sequence, and a sequence coding for a polypeptide nearly identical to the human immunoglobulin heavy chain variable domain polypeptide sequence described by Huang

and Stollar (*J. Immunol.* 151: 5290-5300, 1993). The coding sequence of the synthetic VH gene segment is designed based on the published sequence of a rabbit VH1 gene (a2, Knight and Becker, *Cell* 60:963-970, 1990) and is more than 80% identical to rabbit VH gene segments. The 5' and the 3' flanking sequences in the synthetic VH segment are derived from the upstream and downstream regions of the a2-allotype rabbit VH1 gene. The synthetic VH gene of SEQ ID NO: 52 is used to replace the rabbit VH1 gene on the BAC clone by homologous recombination using the pET or the redαβγ system. The modified BAC clone is amplified and purified using standard procedures.

Example 7

Construction of a DNA Fragment Containing a Portion of a Rabbit Light Chain Locus with a Human Cκ Gene Segment and a VJ Gene Segment Encoding a Human VL Domain Polypeptide Sequence

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit light chain Cκ1 gene from a b5-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 53) is generated by PCR using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit Cκ1 gene, the human Cκ1 gene, and a sequence derived from the 3' flanking region of the rabbit Cκ1 gene (Figure 10).

A genomic BAC library derived from a b5-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit Cκ1. A BAC clone containing rabbit light chain gene segments is identified. The rabbit Cκ1 gene on this BAC clone is replaced with the human Cκ1 gene on the DNA fragment of SEQ ID NO: 53 by homologous recombination in *E.coli* using the pET or the redαβγ system. This replacement is accomplished by two consecutive recombination steps: first the rabbit Cκ1 gene segment is replaced with a marker gene; then the marker gene is replaced the human Cκ1 gene segment.

The modified BAC clone containing rabbit light chain genes and the inserted human Cκ1 gene is further modified by inserting a rearranged VJ DNA fragment into the

J region of the rabbit light chain locus. The rearranged VJ DNA fragment encodes a human immunoglobulin variable domain polypeptide described by Pritsch et al. (*Blood* 82(10):3103-3112, 1993) and Lautner-Rieske et al. (*Eur. J. Immunol.* 22 (4), 1023-1029, 1992)) (Figure 7). The nucleotide sequence of the rearranged VJ fragment is designed to maximize the sequence homology at the nucleotide level to the rabbit Vkappa sequence published by Lieberman et al. (*J. Immunol.* 133 (5), 2753-2756, 1984). This rearranged VJ DNA sequence is more than 80% identical with known rabbit Vκ genes. Using overlapping oligonucleotides in PCR, the rearranged VJ DNA fragment is linked to a 5' and a 3' flanking sequence, resulting the DNA fragment of SEQ ID NO: 54 (Figure 11). The 5'flanking sequence is derived from 5' of a rabbit Vκ, the 3'flanking sequence is derived from 3' of rabbit J2. The DNA fragment of SEQ ID NO: 54 is subsequently inserted into the rabbit light chain locus by homologous recombination in *E. coli* using the pET or the redeβγ system. The insertion is performed in such a way that the rabbit light chain region containing the rabbit Vκ1 gene segment, the rabbit J1 and J2 segments, and the sequences in between, is replaced with the rearranged VJ DNA fragment. Again, this insertion is accomplished by replacement of the rabbit V to J region with a marker gene, followed by the replacement of the marker gene with the rearranged VJ DNA fragment. The modified BAC clone is amplified and purified using standard procedures.

Example 8

Transgenic Rabbits Expressing the Humanized Immunoglobulin Light and/or Heavy Chain Transgene

Transgenic rabbits are generated as described by Fan et al. (*Pathol. Int.* 49: 583-594, 1999). Briefly, female rabbits are superovulated using standard methods and mated with male rabbits. Pronuclear-stage zygotes are collected from oviduct and placed in an appropriate medium such as Dulbecco's phosphate buffered saline supplemented with 20% fetal bovine serum. The exogenous DNA (e.g., the humanized BAC clone from Example 4 and/or 5 which has been linearized prior to injection) is microinjected into the male pronucleus with the aid of a pair of manipulators. Morphological surviving zygotes

are transferred to the oviducts of pseudopregnant rabbits. Pseudopregnancy is induced by the injection of human chorionic gonadotrophin (hCG). Between about 0.1-1% of the injected zygotes develop into live transgenic rabbits. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene.

cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) of a transgenic rabbit. Primers specific for the human transgene (human CH gene segment or the synthetic humanized VH gene segment) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is transcribed in the animal. Amplified products are sequenced and the presence of donor sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic founder rabbits is determined using an ELISA assay.

Example 9

Production of Humanized Antibodies From Transgenic Rabbits with the Genetic Background of the Alicia and/or Basilea Rabbit Strain

The Alicia strain lacks the VH1 gene segment and therefore has an impaired Ig heavy chain expression. Transgenic founder rabbits capable of expressing humanized heavy chain molecules in the genetic background of the Alicia rabbit strain are generated, e.g., by using fetal fibroblasts established from Alicia rabbits in Examples 4-5 above, or by using zygotes from female Alicia rabbits mated with male Alicia rabbits in Example 8 above. Transgenic animals are also obtained which are homozygous for the Alicia Ig phenotype and are also homozygous for a humanized heavy chain transgene. Serum is tested in ELISA for the presence of humanized heavy chain (e.g., a human heavy chain

constant region). The concentration of antibodies with humanized Ig heavy chains in these homozygous Alicia animals is substantially higher, e.g., about 10 to 100 fold higher, than that produced from a transgene integrated in the genome of wild type (non-Alicia) rabbits.

The Basilea strain does not express κ1 light chain and in its place exclusively express the κ2 and λ light chains. Transgenic founder rabbits capable of expressing humanized light chain molecules in the genetic background of the Basilea rabbit strain are generated, e.g., by using fetal fibroblasts established from Basilea rabbits in Examples 4-5 above, or by using zygotes from female Basilea rabbits mated with male Basilea rabbits in Example 8 above. Transgenic animals are obtained which are homozygous for the Basilea light chain phenotype, and are also homozygous for a humanized light chain transgene. Serum is tested in ELISA for the presence of the humanized light chain. The concentration of the humanized light chain in the homozygous Basilea animals is substantially higher, about 10-100 fold higher, than the concentration of a humanized light chain in a transgenic rabbit with the wild type (non-Basilea) genetic background. Transgenic founder rabbits are mated with each other to generate transgenic rabbits with the following traits: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, (3) homozygous for the Alicia heavy chain locus, and (4) homozygous for the Basilea light chain locus.

Example 10

Construction of a DNA Fragment Containing a Modified Chicken Light Chain Locus Having a Human Clambda2 Gene Segment and a VJ Gene Segment Encoding a Human VL Domain

A genomic BAC library derived from a jungle fowl chicken was screened with radiolabeled probes specific for chicken light chain Clambda and chicken Vpsi25 (the V gene segment at the very 5' end of the light chain locus). A BAC clone containing the entire lambda light chain locus was identified. The chicken Cλ gene on this BAC clone is replaced with the human Cλ2 gene by homologous recombination in *E.coli* using the pET system (Zhang et al., *Nat. Biotechnol.* 18(12):1314-7, 2000) as follows.

A first DNA fragment containing a kanamycin selection cassette was generated by PCR using primers specific for Tn5 gene. The 5' primer (5'catacacagccatacatacgcgtgtggccgctctgcctctctcttgcaggTATGGACAGCAAGCGAACCG 3', SEQ ID NO: 55) was designed to include 50 bp at the 5' end (lower case), derived from the 5' flanking region of the chicken light chain Cλ gene. The 3' primer (5'atcagggtgacccctacgttacactcctgtcaccaaggagtgggagggacTCAGAAGAACTCGTCAAGA AG3', SEQ ID NO: 56) was designed to include about 50 bp at the end (lower case), derived from the 3' flanking region of the chicken light chain Cλ gene.

A second DNA fragment (SEQ ID NO: 57) was synthesized using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the chicken light chain Clambda gene, the human Clambda2 gene, and a sequence derived from the 3' flanking region of the chicken Clambda gene (Figure 12).

E. coli cells of the chicken light chain BAC clone were transformed with a recombination plasmid expressing the recE and recT functions under an inducible promotor. Cells transformed with the recombination plasmid were then transformed with the first DNA fragment above and selected afterwards in media containing kanamycin. Clones resistant to kanamycin were identified, and the replacement of the chicken Cλ segment by the kanamycin selection cassette via homologous recombination was confirmed by restriction enzyme digest.

In the second homologous recombination step, cells positive for the kanamycin selection cassette were transformed with the second DNA fragment above. Transformed cells were screened for the loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human Cλ2 gene. The exchange was confirmed by restriction enzyme digest and/or sequence analysis.

The ET cloning procedure is summarized in Figure 13.

The BAC clone containing the chicken light chain locus and the inserted human Clambda2 gene segment was further modified by inserting a rearranged VJ DNA fragment. The rearranged VJ DNA fragment encodes a human immunoglobulin variable domain polypeptide described by Kametani et al. (*J. Biochem.* 93 (2), 421-429, 1983) as

IG LAMBDA CHAIN V-I REGION NIG-64 (P01702) (Figure 14). The nucleotide sequence of the rearranged VJ fragment was so designed as to maximize the sequence homology at the nucleotide level to the chicken Vlambda1 sequence published by McCormack et al. (*Cell* 56, 785-791, 1989). This rearranged VJ DNA sequence is more than 80% identical with known chicken light chain V genes. The rearranged VJ DNA fragment was linked to a 5' flanking sequence and a 3' flanking sequence, resulting in the DNA fragment of SEQ ID NO: 58 (Figure 14). The 5' flanking sequence was derived from 5' of chicken Vlambda1, and the 3' flanking sequence was derived from 3' of chicken J. The DNA fragment of SEQ ID NO: 58 was subsequently inserted into the chicken light chain locus in *E. coli* using the pET system as shown in Figure 15. The insertion was performed in such a way that the region on the chicken light chain locus from the 5' end of the chicken Vlambda1 gene segment to the 3' end of the chicken J region was replaced with the rearranged, synthetic VJ DNA fragment. Again, this insertion wais accomplished by the replacement of the chicken V-J region with a marker gene, followed by the replacement of the marker gene with the rearranged VJ DNA fragment. The modified region of the chicken light chain locus is shown in Figure 15. The modified BAC clone was amplified and purified using standard procedures.

Example 11

Construction of a DNA Fragment Containing a Portion of a Chicken Heavy Chain Locus With a Human Cγ1 Gene Segment and a VH Gene Segment Encoding a Human VH Domain Polypeptide Sequence

A jungle fowl chicken genomic BAC library was generated by standard procedures and screened with probes specific for chicken Cγ. A BAC clone containing chicken heavy chain gene segments is identified. The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the heavy chain Cγ gene are sequenced. The chicken Cγ gene on this BAC clone is replaced with the human Cγ1 gene by homologous recombination in *E.coli* using the pET system as follows.

A first DNA fragment containing a kanamycin selection cassette is generated by PCR using primers specific for Tn5 gene. The 5' and 3' primers are designed to

include about 50 bp at the end, derived from the 5' and 3' flanking regions of the chicken heavy chain Cγ gene.

A second DNA fragment is generated by PCR using overlapping oligonucleotides wherein this second DNA fragment contains from 5' to 3', a sequence of about 50 bp derived from the 5' flanking region of the chicken Cγ gene, the human Cγ1 gene, and a sequence of about 50 bp derived from the 3' flanking region of the chicken Cγ gene.

E. coli cells of the chicken CY BAC clone are transformed with a recombination plasmid expressing the recE and recT functions under an inducible promotor. Cells transformed with the recombination plasmid are further transformed with the first DNA fragment and selected in media containing kanamycin. Clones resistant to kanamycin are identified, and the replacement of the chicken CY segment by the kanamycin selection cassette via homologous recombination is confirmed by restriction enyme digest.

In the second homologous recombination step, cells positive for the kanamycin selection cassette are now transformed with the second DNA fragment described above. Transformed cells are screened for loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human Cγ1 gene. The exchange is confirmed by restriction enzyme digest and/or sequence analysis.

The BAC clone containing the inserted human Cγ1 gene is further modified by replacing the 3'proximal VH1 segment (i.e., the 3'proximal VH1 gene in the V region) with a synthetic VH gene segment. This synthetic VH gene segment is designed based on the published sequence of a chicken VH1 gene (Arakawa et al., EMBO J 15(10): 2540-2546, 1996). The synthetic gene segment is more than 80% identical to chicken VH gene segments and encodes an amino acid sequence that is identical to the amino acid sequence of a human immunoglobulin heavy chain variable domain polypeptide described by Matthyssens and Rabbitss (in Steinberg CM and Lefkovits I, (eds). *The Immune System*: 132-138, S. Karger, NY 1981). This synthetic VH segment including 5' and 3' flanking sequences is synthesized by PCR using overlapping oligonucleotides. The 5' and the 3' flanking sequences are derived from the upstream and downstream regions of chicken

VH1 gene. This synthetic VH segment is used to replace the chicken VH1 gene on the BAC clone by homologous recombination using the pET system. The modified BAC clone is amplified and purified using standard procedures.

Example 12

Transgenic Chicken Expressing the Humanized Immunoglobulin Light and/or Heavy Chain Transgenes

The production of transgenic chicken is carried out using techniques as described by Etches et al., *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and potential applications", *Trends Biotechnol 12:415* (1994); and in WO 200075300, "Introducing a nucleic acid into an avian genome, useful for transfecting avian blastodermal cells for producing transgenic avian animals with the desired genes, by directly introducing the nucleic acid into the germinal disc of the egg".

Briefly, the modified BAC clones are linearized and mixed with a transfection reagent to promote uptake of DNA into cells. The formulations are injected into a multicell stage chicken embryo in close proximity to the germinal disc. The window in the egg shell is closed and the eggs are incubated. After hatching chimeric chickens are identified by PCR and Southern blot analysis using transgene specific sequences. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene. Heavy and light chain transgenic animals are bred with each other to generate transgenic chickens expressing antibodies having humanized heavy and light chains.

cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) from transgenic chickens. Primers specific for the human transgene (e.g., human CH gene segments and/or the synthetic humanized VH gene segments) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is transcribed in the animal. Amplified products are sequenced and the presence of donor

sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic chickens is determined using an ELISA assay.

Example 13

Production of Functional Humanized Antibodies in Transgenic Chicken with the Agammaglobulinemic Phenotype

Transgenic chickens with the following traits are produced: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, and (3) homozygous for the agammaglobulinemic phenotype. These animals produce antibodies into the blood and eggs, and antibodies can be purified from either source. In general, antibody concentrations in the eggs are about 5% to 50% of antibodies concentration in the blood. Animals that contain humanized antibodies at high levels in eggs can be selected and bred to produce offspring. Alternatively, transgenic animals can be generated that specifically secrete humanized antibodies into their eggs.

Example 14

Generation Of Transgenic Chickens Expressing Humanized Immunoglobulin

Chicken embryonic stem cells are isolated and cultured as described by Pain et al. (*Development* 122, 2339-2348; 1996). Chicken embryos are obtained from eggs immediately after they are laid. The entire blastoderm is removed by gentle aspiration, embryos are slowly dissociated mechanically and cells are seeded in ESA complete medium on inactivated STO feeder cells. ESA medium is composed of MEM medium containing 10% FCS, 2% chicken serum, 1% bovine serum albumin, 10 ng/ml ovalbumin, 1 mM sodium pyruvate, 1% non-essential amino acids, 1 μM of each nucleotide

adenosine, guanosine, cytidine, uridine, thymidine, 0.16 mM β-mercaptoethanol, ESA complete medium is supplemented with 10 ng/ml bFGF, 20 ng/ml h-IGF-1, 1% vol/vol avian-SCF and 1% vol/vol h-LIF, 1% vol/vol h-IL-11. Cell cultures are incubated wt 37°C in 7.5 CO_2 and 90% humidity. After 48 hours fresh blastodermal cells are added to the culture in half of the original volume of ESA complete medium. After an additional incubation for three days, the culture medium is partially (50%) replaced with fresh ESA complete medium, and totally every day thereafter. For cell harvesting, cultures are washed with PBS and incubated in a pronase solution (0.025% w/v). Dissociated cells are transfected with various linearized transgenic constructs containing a humanized Ig locus. Transfected cells are incubated with STO feeder cells (as described above) in the presence of selective antibiotics. Cells are transferred onto fresh feeder cells twice per week. Antibiotic resistant cells are isolated and the integration of a humanized Ig gene fragments at a random site or at the corresponding chicken immunoglobulin gene loci is confirmed by PCR.

Subsequently, genetically modified cells are injected into recipient embryos. As recipient embryos, freshly laid eggs are irradiated (6Gy - Cobalt source). Between 100 to 200 genetically modified cells are injected into the subgerminal cavity using a micropipet. The window in the egg shell is closed and the eggs are incubated. Somatic chimerism of hatched chickens is evaluated by PCR. Germ-line chimerism is assessed by mating of somatic chimeras.

Example 15

Immunization Of Transgenic Animals

Genetically engineered chickens are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (5μg in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 μg/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μl/well). Chicken serum is diluted in PBS/1%NFM and added to the

coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is used. Serum from non-immunized chickens does not react with HBsAg. At a dilution of 1:250 the optical density measured in uncoated and HBsAg coated wells is below 0.2. In contrast, serum from immunized chickens contains humanized antibodies reactive with HBsAg. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered chickens produce humanized anti-HBsAg antibodies following immunization.

Genetically engineered rabbits are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (10μg in incomplete Freund's adjuvant) on day 0 and day 14. On day 28 animals are bled from the ear and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 μg/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μl/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horse-radish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control serum from non-immunized rabbits is used. Serum from non-immunized rabbits does not react with HBsAg. At a dilution of 1:100 the optical density measured in uncoated and HBsAg coated wells is below 0.4. In contrast, serum from immunized rabbits contains partially human antibodies reactive with HBsAg. At a serum dilution of 1:100 the measured optical density is 2.8. Upon further dilution of the serum the measured optical density declines to 0.2 (at a dilution of 25600). No antibodies reactive

with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce humanized anti-HBsAg antibodies following immunization.

Example 16

Complement Mediated Cytotoxicity of Virus Infected Cell Line Using Humanized Antibodies

A human liver carcinoma cell line expressing HBsAg is labeled with 0.1 mCi ^{51}Cr in 100 ul PBS for 1 hr at 37°C. Two thousand ^{51}Cr-lableled cells are incubated with serum from genetically engineered rabbits or chickens expressing anti-HbsAg humanized immunoglobulins. After two hours at 37°C the release of ^{51}Cr into the supernatant is determined by measuring radioactivity using a scintillation counter. For the determination of maximum release, 1% Triton X100 is added. The degree of cell lysis is calculated as follows: %Lysis = CPM experimental ±CPM#spontaneous / CPM# total ± CPM spontaneous. Incubation of labeled cells with serum (diluted 1:30) from non-immunized rabbits does not result in cell lysis (<10%). However, incubation of cells with serum from immunized rabbits causes 80% cell lysis. Inactivation of complement in the serum by heat treatment (56°C for 30 minutes) renders the serum from immunized rabbits inactive. These results demonstrate that humanized antibodies produced by genetically engineered rabbits bind to HBsAg-positive cells and cause complement dependent lysis.

Example 17

Immunization of Transgenic Animals against Staphylococcus aureus

Genetically engineered chickens are immunized intramuscularly with a recombinant fragment of the Staphylococcus aureus collagen adhesin protein (100μg in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 μg/ml collagen adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μl/well).

Chicken serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is used. Serum from non-immunized chickens does not react with collagen adhesin protein. At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin protein coated wells is below 0.2. In contrast, serum from immunized chickens contains humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered chickens produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

Genetically engineered rabbits are immunized intramuscularly with recombinant fragment of the Staphylococcus aureus collagen adhesin protein (100μg in incomplete Freund's adjuvant) on day 0 and day 14. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 μg/ml collagen adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μl/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized rabbit is used. Serum from non-immunized rabbits does not react with collagen adhesin protein. At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin protein coated wells is below 0.2. In contrast, serum from immunized rabbits contains

humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

Example 18

Protection Against *Staphylococcus Aureus* Infection In A Mouse Model

Naive mice are passively immunized i.p. on day -1 with 16 mg of the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein (from Example 17) or with the immunoglobulin fraction from non-immunized animals. On day 0, the mice are challenged i.v. with $4x10^7$ CFU *S. aureus* per mouse and mortality is monitored over the next 7 days. Mortality rate in the control groups is 80% and 10% in the group treated with the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein. The data indicate that anticollagen adhesin antibodies can protect mice against lethal *S. aureus* challenge.

Example 19

Antigen-Specific Hybridomas Made From Transgenic Animals.

Transgenic animals are immunized with an antigen (e.g., KLH, human red blood cells or sheep red blood cells). Spleen cells are removed at various times after immunization and fused with myeloma cell lines derived from rabbit and chicken, respectively. After fusion cells are plated into 96 well plates and supernatants are tested for the presence of humanized antibodies. To demonstrate that the antibodies contain human immunoglobulin sequences, hybridomas are stained with fluorescent-labeled antibodies reactive with human heavy and light chain immunoglobulins. Limiting dilution is conducted to purify hybridomas to monoclonality.

Example 20

Evaluation of Immunogenicity

Serum samples are collected from five cynomologous monkeys on day 0. Subsequently, a purified partially human polyclonal antibody preparation (5 mg/kg) is administered into five cynomologous monkeys by intravenous administration. The administration is repeated six times in bi-weekly intervals. Monkeys are monitored closely for any side-effects (e.g., anaphylactic shock, reflected by an elevated body temperature). After seven months serum is collected from blood samples. Affinity resins containing purified human IgG or partially human IgG are produced by standard procedure using CNBr-activated Sepharose. Monkey serum samples (3 ml) are added to the IgG-affinity resin (4 ml) containing 10 mg human or partially human IgG. Subsequently, the columns are washed with PBS. Bound monkey immunoglobulin is eluted from the column with 0.1M glcyin/HCl pH2.5 and dialyzed 2 times against PBS. The protein content of the eluted fractions is determined using the BCA assay using human IgG as a standard. The total amounts of protein in these fractions demonstrate that therapy with partially human IgG does not lead to a significant antibody response in the treated animals.

Example 21

Treating Animals Using Humanized Antibodies

Humanized polyclonal immunoglobulins are purified from the serum of genetically engineered rabbits, or from egg yolk of genetically engineered chickens, by ammonium sulfate precipitation and ion exchange chromatography. SCID-mice are injected with one million human liver carcinoma cells expressing HBsAg. Subsequently, 25 µg immunoglobulin is injected peritoneally once per day. Animals treated with antibodies isolated from non-immunized rabbit serum die after about 60 days. This is similar to untreated recipients of liver carcinoma cells. In contrast, mice treated with antibodies isolated from immunized rabbit serum survive for more than 150 days. This

demonstrates that human antibodies produced in genetically engineered rabbits are capable of eliminating human carcinoma cells from SCID-mice.

What is claimed is:

1. An isolated nucleic acid molecule comprising the sequence as set forth in any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13, or a portion of any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13.

2. A recombination vector for replacing an Ig gene segment from a non-human animal with a human Ig gene segment, comprising from 5' to 3', a 5' nucleotide sequence, said human Ig gene segment, and a 3' nucleotide sequence, wherein said 5' nucleotide sequence and said 3' nucleotide sequence are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.

3. The recombination vector of claim 2, wherein said non-human animal is an animal which relies primarily on gene conversion in generating antibody diversity.

4. The recombination vector of claim 3, wherein said animal is rabbit, pig, chicken, sheep or cow.

5. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain constant region.

6. The recombination vector of claim 5, wherein said vector comprises from 5' to 3', a 5' nucleotide sequence as set forth in any one of SEQ ID NO: 12, SEQ ID NO: 13, a portion of SEQ ID NO: 12, or a portion of SEQ ID NO: 13; a human heavy chain constant region gene segment; a 3' nucleotide sequence as set forth in SEQ

ID NO: 10 or a portion of or SEQ ID NO: 10; and wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.

7. The recombination vector of claim 5, comprising the nucleotide sequence as set forth in SEQ ID NO: 51 wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.

8. The recombination vector of claim 5, wherein said vector is useful for replacing a rabbit light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 53.

9. The recombination vector of claim 5, wherein said vector is useful for replacing a chicken light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 57.

10. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain variable region.

11. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit heavy chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 52.

12. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit light chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 54.

13. A transgenic vector comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene

segments is a human Ig gene segment, wherein said gene segments are juxtaposed in an unrearranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

14. The transgenic vector of claim 13, wherein said non-human animal is an animal which generates antibody diversity substantially by gene conversion.

15. The transgenic vector of claim 14, wherein said non-human animal is rabbit, pig, chicken, sheep or cow.

16. The transgenic vector of claim 13, wherein said humanized Ig locus is a heavy chain locus and comprises at least one V gene segment, at least one D gene segment, at least one J gene segment and at least one constant region gene segment.

17. The transgenic vector of claim 16, wherein said constant region gene segment is a human heavy chain constant region gene segment.

18. The transgenic vector of claim 17, wherein said human heavy chain constant region gene segment is a Cγ.

19. The transgenic vector of claim 17, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.

20. The transgenic vector of claim 19, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.

21. The transgenic vector of claim 13, wherein said humanized Ig locus is a light chain locus and comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment.

22. The transgenic vector of claim 21, wherein said constant region gene segment is a human light chain constant region gene segment.

23. The transgenic vector of claim 22, wherein said human light chain constant region gene segment is Cλ or Cκ.

24. The transgenic vector of claim 22, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.

25. The transgenic vector of claim 24, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.

26. The transgenic vector of claim 22, wherein said human V gene segment is placed immediately 5' to a J gene segment in a rearranged configuration.

27. A method of making a transgenic vector comprising a humanized Ig locus capable of producing a functional repertoire of humanized antibodies in a non-human animal, comprising:

(i) obtaining a DNA fragment comprising an Ig locus or a portion thereof from said non-human animal which comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment; and

(ii) integrating at least one human Ig gene segment into said DNA fragment of step (i) to produce a humanized Ig locus, wherein said human Ig

gene segment is linked to the sequences of non-human origin operably as to permit gene rearrangement and gene conversion of said humanized Ig locus and the production of a functional repertoire of humanized antibodies in said non-human animal.

28. The method of claim 27, wherein the integration of said human Ig gene segment is achieved by homologous recombination, thereby replacing an Ig gene segment in said Ig locus or said portion thereof from said non-human animal.

29. The method of claim 28, wherein the homologous recombination is achieved in a bacterial cell, a yeast cell, or a non-human animal cell.

30. The method of claim 28, wherein the human Ig gene segment is provided on a recombination vector, and is linked to a 5' nucleotide sequence and a 3' nucleotide sequence which are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.

31. A transgenic animal comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene segments is a human Ig gene segment, said gene segments being juxtaposed in an unrearranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

32. The transgenic animal of claim 31, wherein said animal is selected from rabbit, pig, chicken, sheep or cow.

33. A B cell from the transgenic animal of claim 31.

34. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized Ig heavy chains, comprising:

(i) introducing a transgenic construct according to any one of claims 16-20 into a recipient cell of a non-human animal and integrating the humanized heavy chain locus in the transgenic construct into the genome of said recipient cell; and

(ii) deriving an animal from the recipient cell having the humanized heavy chain locus integrated in the genome, thereby producing a functional repertoire of humanized Ig heavy chains.

35. The method of claim 34, wherein said animal is rabbit and said recipient cell is a cell in an early embryo.

36. The method of claim 35, wherein said rabbit has an impaired expression of endogenous Ig molecules.

37. The method of claim 34, wherein said animal is chicken and said recipient cell is a fertilized egg.

38. The method of claim 37, wherein said chicken has an impaired expression of endogenous Ig molecules.

39. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized Ig light chains, comprising:

(i) introducing a transgenic construct according to any one of claims 21-26 into a recipient cell of a non-human animal and integrating the humanized light chain locus in the transgenic construct into the genome of said non-human animal; and

(ii) deriving an animal from the recipient cell having the humanized light locus integrated in the genome, thereby producing a functional repertoire of humanized Ig light chains.

40. The method of claim 39, wherein said animal is rabbit and said recipient cell is a cell in an early embryo.

41. The method of claim 40, wherein said rabbit has an impaired expression of endogenous Ig molecules.

42. The method of claim 39, wherein said animal is chicken and said recipient cell is a fertilized egg.

43. The method of claim 42, wherein said chicken has an impaired expression of endogenous Ig molecules.

44. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies, comprising:

(i) introducing a transgenic construct according to any one of claims 16-20 and a transgenic construct according to any one of claims 21-26 into a recipient cell of a non-human animal, and integrating the humanized Ig loci in the transgenes into the genome of said non-human animal; and

(ii) deriving an animal from the recipient cell having the humanized Ig loci integrated in the genome, thereby producing a functional repertoire of humanized antibodies.

45. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies, comprising

(i) making a transgenic non-human animal capable of producing a functional repertoire of humanized heavy chains;

(ii) making a transgenic non-human animal capable of producing a functional repertoire of humanized light chains; and

(iii) mating the transgenic non-human animal of (i) with the transgenic animal of (ii); and

(iv) selecting an offspring which produces both humanized heavy chains and humanized light chains thereby obtaining a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies.

46. A humanized immunoglobulin produced using the transgenic animal of claim 31.

47. A humanized immunoglobulin derived from a transgenic animal, comprising at least a portion of a human imunglobulin polypeptide sequence.

48. The humanized immunoglobulin of claim 47, wherein said transgenic animal generates antibody diversity by gene conversion and/or hypermutation

49. The humanized immunoglobulin of claim 48, wherein said transgenic animal is a rabbit, chicken, sheep or cow.

50. The humanized immunoglobulin of claim 49, wherein said human immunglobulin polypeptide sequence is a heavy chain or light chain polypeptide sequence.

51. The humanize immunoglobulin of claim 50, wherein said portion of a human immunglobulin polypeptide sequence is a human constant region polypeptide sequence.

52. The humanized immunoglobulin of claim 51, wherein said human constant region polypeptide sequence is $C\gamma$, $C\kappa$, or $C\lambda$.

53. The humanized immunoglobulin of claim 51, wherein said portion of a human immunoglobulin polypeptide sequence further comprising a human V domain polypeptide sequence.

54. The humanized immunoglobulin of claim 47, wherein said humanized immunoglobulin is specific for an antigen.

55. The humanized immunoglobulin of claim 54, wherein said antigen is a microorganism selected from bacterium, fungus, or virus; an antigenic portion of said organism; an antigenic molecule derived from said microorganism; or a tumor-associated antigen.

56. The humanized immunoglobulin of claim 55, wherein said bacterim is selected from *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, or *Klebsiella pneumoniae*.

57. The humanized immunoglobulin of claim 55, wherein said fungus is selected from Candida albicans, Candida parapsilosis, Candida tropicalis, or Cryptococcus neoformans.

58. The humanized immunoglobulin of claim 55, wherein said virus is selected from respiratory synctial virus (RSV), Hepatitis C virus (HCV), Hepatits B virus (HBV), cytomegalovirus (CMV), EBV, or HSV.

59. The humanized immunoglobulin of claim 55, wherein said antigen is selected from Her-2-neu antigen, CD20, CD22, CD53, prostate specific membrane antigen (PMSA), or17-1A molecule.

60. An antibody preparation, comprising the humanized immunoglobulin of any one of claims 46-55.

61. The antibody preparation of claim 60, wherein said preparation is a monoclonal antibody preparation.

62. The antibody preparation of claim 60, wherein said preparation is a polyclonal antibody preparation.

63. The antibody preparation of claim 62, wherein said preparation is substantially non-immunogenic to human.

64. A pharmaceutical composition, comprising a pharmaceutically acceptable carrier and the antibody preparation of claim 60.

65. A method of treating a disease in a human subject comprising administering to said subject a thereapeutically effective amount of the antibody preparation of claim 60.

66. The method of claim 59, wherein said disease is caused by bacterial, fungal or viral infection, or said disease is a cancer.

Figure 1(a)-(d). Novel nucleotide sequences 3'prime of the cow Cgamma gene (Cow Cγ 3' flanking sequences). Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M1. The sequences of clone 11, clone 3, and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

```
           1                                                    50
clone11    [shaded primer]CACGA AACTTTACGG
clone3     [shaded primer]CACGA GGCCCTGCAC
clone5     [shaded primer]CACGA GGCCCTGCAC

         151                                                   200
clone11  AATCACTACA AAGAGAAGTC CACCTCGAGG TCTCCGGGTA AATGAGCCTC
clone3   AATCACTACA CGCAGAAGTC CACCTCTAAG TCTGCGGGTA AATGAGCCTC
clone5   AATCACTACA CGCAGAAGTC CACCTCTAAG TCTGCGGGTA AATGAGCCTC

         201                                                   250
clone11  GCGCCGCTGA TCTAGTGGAC GTTCCCTCAT CCACCCACCC CTCCCCCCAC
clone3   ACGTCCCTGC ACCAGCAAGC CCTCACCCAG C..... ..CCACCCTC
clone5   ACGTCCCTGC ACCAGCAAGC CCTCACCCAG C..... ..CCACCCTC

         251                                                   300
clone11  CCCGGGCTCC AGGTCCAGCC AGGGCGCCCT AGCCCCTCCC TGTGTGCATT
clone3   CCCGGGCTCC AAGTCCAGCC AGGACGCCCT AGCCCCTCCC TGTGTGCATT
clone5   CCCGGGCTCC AGGTCCAGCC AGGACGCCCT AGCCCCTCCC TGTGTGCATT

         301                                                   350
clone11  CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA
clone3   CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCACCCTG GGACCCTGCA
clone5   CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA

         351                                                   400
clone11  ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG
clone3   ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG
clone5   ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG

         401                                                   450
clone11  CGGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG
clone3   CAGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG
clone5   CGGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG

         451                                                   500
clone11  GAGCTGACCC CACTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC
clone3   GAGCTGACCC CGCTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC
clone5   GAGCTGACCC CACTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC

         501                                                   550
clone11  ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCACAGCTC
clone3   ACCTGGGGGC TGCCCGAGGC CACGGAGGGG CTTGGCCAGA GGCGTACCTC
clone5   ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCACAGCTC
```

Figure 1(a)

```
          551                                                       600
clone11   CACGGTCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC
clone3    CACGGCCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC
clone5    CACGGTCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC

          601                                                       650
clone11   GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC
clone3    GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC
clone5    GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC

          651                                                       700
clone11   TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
clone3    TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
clone5    TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC

          701                                                       750
clone11   TGTCCTTGGG TGTCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC
clone3    TGTCCCTGGG TGCCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC
clone5    TGTCCTTGGG TGTCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC

          751                                                       800
clone11   CACGCATGGC CAGGGGGTGG CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA
clone3    CACGCGTGGC CGGGGGATGG CCCTGCTCCA CCCTCAGGCT CCCAAGGTCA
clone5    CACGCATGGC CAGGGGGTGG CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA

          801                                                       850
clone11   GGCCTCGCCC TCCCTCGGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
clone3    GGCCTCGCCC TCCCTCAGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
clone5    GGCCTCGCCC TCCCTCGGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG

          851                                                       900
clone11   GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT
clone3    GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGCT
clone5    GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT

          901                                                       950
clone11   CCAGGCCGAG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGT
clone3    CCAGGCCGGG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGC
clone5    CCAGGCCGAG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGT

          951                                                      1000
clone11   GGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA
clone3    AGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA
clone5    GGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA

          1001                                                     1050
clone11   CGAGACAGCC CGCACCCAGC CTCCTCCACA CGCACTCAGG TGTACATGCG
clone3    CAAGACAGCC CGCACCCAGC CTCCTCCACA CGCACTCAGG TGTGCATCCG
clone5    CGAGACAGCC CACACCCCGC CTCCTCCACA CGCACTCAGG TGTGCATCCG
```

Figure 1(b)

```
          1051                                                      1100
clone11   CACATGAGCA CACTTCACCC CGTCACACCC ACACACCTAC ACACACTCAG
 clone3   CACATGAGCA CACTTCACCC CGTCACACCC ACACGCCTAC ACACACTCAG
 clone5   CACATGAGCA CACTTCACCC CATCACACCC ACACGCCTAC ACACACTCAG

          1101                                                      1150
clone11   GTCTCGCACT CGGGGACCCA TGGGGTGACC CCACGGGCCC AGA.CCAGAG
 clone3   GTCTCGCACT CGGGGACCCA TGGGGTGACC CCACAGGCCC AGACCCAGAG
 clone5   GTCTCGCACT CGGGGACCCA TGGGGTGACC CCACAGGCCC AGACCCAGAG

          1151                                                      1200
clone11   CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGGCC CCACCCTCCA
 clone3   CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGTCC CCACCCTCCA
 clone5   CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGTCC CCACCCTCCA

          1201                                                      1250
clone11   GCGCCCATGG GCTGCTCAGC GGCCCTTTCC CACACTGACC ACACTGACCA
 clone3   GCGCCCATGG GCTGCTCAGT GGCCCTTTCC CACACTGACC ACACTGACCA
 clone5   GCGCCCGTGG GCTGCTCAGC GGTCCTTTCC CACACTGACC ACACTGACCA

          1251                                                      1300
clone11   GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GACACCCACG CCCCTCCCTA
 clone3   GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GGCACCCACG CCCCTCCCTA
 clone5   GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GGCACCCATG CCCCTCCCTA

          1301                                                      1350
clone11   GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT CCTGGCAGCC TCACCCCTCG
 clone3   GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT CCTGGCACCC TCACCCCTCA
 clone5   GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT CCTGGCAGCC TCACCCCTCA

          1351                                                      1400
clone11   GGCACAGCAC CCCTCAGGCC CGGTGCTGTC AGCCCTCCCT CCCCGGGGGC
 clone3   GGCACAGGGA CAC...AGCC CGGGGCTGTC TGCCCTCCCT CCCTGGGGGC
 clone5   GGCACAGGGA CAC...AGCC CGGTGCTGTC TGCCCTCCCT CCCTGGGGGC

          1401                                                      1450
clone11   AGGGCCCAGG AACGTGCGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC
 clone3   AGGGCCCAGG CTCACATGCT CTGCTGACCC TCCCGGCTCC AGGCCTGGCC
 clone5   AGGGCCCAGG CTCACATGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC

          1451                                                      1500
clone11   CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCCTGT GGGGAGGTAG
 clone3   CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCCTGG GGGGAGGTGG
 clone5   CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCCTGG GGGGAGGTGG

          1501                                                      1550
clone11   GGTCAGGGTC CCAGCTCAGG GCACAGCTCA GGATGGGAGC AGGACCCCAC
 clone3   GGTCAGGGCC CCAGCTCAGG GCACAGCTCA GGATGGGAAC AGGACACCAC
 clone5   GGTCAGGGCC CCAGCTCAGG GCACAGCTCA GGATGGGAGC AGGACACCAC
```

Figure 1(c)

```
          1551                                                      1600
clone11   AGGCCAGGCC CAGATAGCAG CCAGGGCTGG AGGGGTTGGG GCTGGGGCTG
clone3    AGGCCAGGCC CAGACAGTGG CCAGGGCTGG AGGGGTGGGG TCTGGGGCTG
clone5    AGGCCAGGCC CAGACAGTGG CCAGGGCTGG AGGGGTGGGG TCTGGGGCTG

          1601                                                      1650
clone11   GGCCCCAGAG ACTGACCTCA GGTGACCCCT GCCTGGCCCA TGGGGAGATC
clone3    GGGCCCAGAG ACTGACCTCA GGTGATCCCT GCCCAGCCCA TGGGGGGATC
clone5    GGCCCCAGAG AATGACCTCA GGTGATCCCT GCCCAGCCCA TGGGGGGATC

          1651                                                      1700
clone11   ACGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCC...T ACCCCAGTGA
clone3    CTGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA
clone5    CTGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA

          1701                                                      1750
clone11   CCCTGCCCAG CCCTCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT
clone3    TGCCACCCAG CCCCCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT
clone5    TGCCACCCAG CCCCCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT

          1751                                                      1800
clone11   GCAGACTTGC TGCTGGAGGA GGAGATCTGT GCGGACGACC TGGATGGGGA
clone3    GCAGACCTGC TGCTGGAGGA GGAGATCTGT GCGGACGCCC AGGACGGGGA
clone5    GCAGACCTGC TGCTGGAGGA GGAGATCTGT GCGGACGCCC AGGACGGGGA

          1801                                                      1850
clone11   GCTGGACGGG CTC[redacted]
clone3    GCTGGACGGG CTC[redacted]
clone5    GCTGGACGGG CTC[redacted]
```

Figure 1(d)

Figure 2(a)-(e). Novel nucleotide sequnces 3'prime of the sheep Cgamma genes. Primers are shown in shaded boxes. The 5′ primer is in CH3, and the 3′ primer is in M2. The sequences of clone 11 and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

```
        101                                            150
clone11 CCTACGCGTG TGTGGTCATG CACGAGGCTC TACACAACCA CTACACACAG
 clone1 CCTACGCGTG TGTGGTCATG CACGAGGCTC TGCACAACCA CTACACACAG

        151                                            200
clone11 AAGTCGATCT CTAAGCCTCC GGGTAAATGA GCCACATGCC CCCGCACCAG
 clone1 AAGTCGGTCT CTAAGCCTCC GGGTAAATGA GCCACACGCC CCCGCACCAG

        201                                            250
clone11 CAAGCCCTCA CCCAGCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG
 clone1 CAAGCCCTCA CCCAGCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG

        251                                            300
clone11 CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA
 clone1 CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA

        301                                            350
clone11 CCCAGGCCGC CCTGGGACCC TGCAACGCTG TGCTTGTTCT TTCCGAGGCA
 clone1 CCCAGGCCGC CCTGGGACCC TGCAGCGCTG TGCTGGTTCT TTCCGAGGCA

        351                                            400
clone11 GAGCCCTGGT GACCGCCAGG CCTGCGGGGG GTGGGCTGAG CCCACTCTGG
 clone1 GAGCCCTGGT GATCGCCAGG CCTGCGGGGG GCGGGCTGAG CCCACTCTGG

        401                                            450
clone11 GCCGCTTGGT TCAGCATCTG TGGGGGCGCT GACCCCTCTC CGGGCCAGAC
 clone1 GCCGCTTGGT TCAGCATCTG TGGGGGCGCT GACCCCTCTC CGGGCCAGAC

        451                                            500
clone11 ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCCC GAGGCCTCGG
 clone1 ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCCC GAGGCCTCGG

        501                                            550
clone11 AGGGGCTTGG CCAGAGGCGC AGCTTCACGG CCCCCTCCAG CCACCACATT
 clone1 AGGGGCTTGG CCAGAGGCGC AGCTCCACGG CCCCCTCCAG CCACCACATT

        551                                            600
clone11 CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCCGAC ACCTCAGGGA
 clone1 CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCCGAC ACCTCAGGGA

        601                                            650
clone11 TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG
 clone1 TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG
```

Figure 2(a)

```
          651                                                       700
clone11   GCCGCGGCCT TGTCCCCAGG CCCCTGTTCC TGGGTGCCCA GAGTCCGTGT
clone1    GCCGCGGCCT TGTCCCCAGG CCCCTGTTCC TGGGTGCCCA GAGTCCGTGT

          701                                                       750
clone11   GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCCAGGGG GAGGCCCTGC
clone1    GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCCAGGGG GAGGCCCTGC

          751                                                       800
clone11   TTCACCCTCA GGCTCCCGAG GTCAGGCATC ATCCTCGTCG GCCAGTAGCT
clone1    TTCACCCTCA GGCTCCCGAG GTCAGGCATC ATCCTCGTCG GCCAGTAGCT

          801                                                       850
clone11   CTGCCTGGCT CTCTCTGCCC GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT
clone1    CTGCCTGGCT CTCTCTGCCC GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT

          851                                                       900
clone11   CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG
clone1    CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG

          901                                                       950
clone11   CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCCAGAGC CCCAGACACC
clone1    CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCCAGAGC CCCAGACACC

          951                                                      1000
clone11   AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA
clone1    AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA

          1001                                                     1050
clone11   CACGCACTCA GGCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA
clone1    CACCCACTCA GGCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA

          1051                                                     1100
clone11   CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG
clone1    CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG

          1101                                                     1150
clone11   TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG
clone1    TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG

          1151                                                     1200
clone11   GACACCAGCT GGTCCCCATT CTCCAGCGCC CTTGGGCTGC TCAGTGGCCC
clone1    GACACCAGCT GGTCCCCATC CTCCAGCGCC CTTGGGCTGC TCAGTGGCCC

          1201                                                     1250
clone11   TTTCCCACAC TGACCACACT GACCAGGTCA GACATCCTTC CTGCCCTCCC
clone1    TTTCCCACAC TGACCACACT GACCAGGTCA GACATCCTTC CTGCCCTCCC
```

Figure 2(b)

```
          1251                                                      1300
clone11   CTGGGGCACC CAGGCCCCTC CCTCGCAGGC TGAGACCCCC CCTCAGCCCC
clone1    CTGGGGCACC CACGCCCCTC CCTTGCAGGC TGAGACCCCC CCTCAGCCCC

          1301                                                      1350
clone11   TCGTCCTGGC ACCCTCACCC CTCGGGCACA GGGACACAGC CCGGCACTGT
clone1    TCGTCCTGGC ACCCTCACCC CTCGGGCACA GGGACACAGC CCGGCACTGT

          1351                                                      1400
clone11   CTGCCCTCCC TCTCGGGGAC AGAGCCCAGG CACGTGTGCT CTGCTGAGCC
clone1    CTGCCCTCCC TCTCGGGGAC AGAGCCCAGG CACGTGTGCT CTGCTGAGCC

          1401                                                      1450
clone11   TCCCGGCTCC AGGCCTGGCC CCCAGGGCAG AGGAGGCCAG GAATTGAGCC
clone1    TCCCGGCTCC AGGCCTGGCC CCCAGGGCAG AGGAGGCCAG GAATTGAGCC

          1451                                                      1500
clone11   TCTGTCCTGC GGGGAGGTGG GGTCAGGGCC CCAGCTCAGG GCACAGCTCA
clone1    TCTGTCCTGC GGGGAGGTGG GGTCAGGGCC CCAGCTCAGG GCACAGCTCA

          1501                                                      1550
clone11   GGATGGGAGC AGGACCCCAC AGGCCAGGCC CAGACAGTGG CCAGGGCTGG
clone1    GGATGGGAGC AGGACCCCAC AGGCCAGGCC CAGACAGTGG CCAGGGCTGG

          1551                                                      1600
clone11   GGCTGGGGCT GGGGCCCAGA GACTGACCTC AGGTGACCCC TGCCCGGCCC
clone1    GGCTGGGGCT GGGGCCCAGA GACTGACCTC AGGTGACCCC TGCCCGGCCC

          1601                                                      1650
clone11   ATGGGGGATC ACACCGCCAT CCCCCCCGCC GCAGAGGGAG CCCTGCCCCG
clone1    ATGGGGGATC ACACCGCCAT CCCCCCCGCC GCAGAGGGAG CCCTGCCCCG

          1651                                                      1700
clone11   AAGCCCCGAT GGCCCCGCCC AGCCCCCCGT GGGCAGACAC AGCACTGACC
clone1    AAGCCCCGAT GGCCCCGCCC AGCCCCCCGT GGGCAGACAC AGCACTGACC

          1701                                                      1750
clone11   CCCCTCCCTG TGCAGATCTG CTGCTGGAGG AGGAGAGCTG TGCGGACGCC
clone1    CCCCTCCCTG TGCAGATCTG CTGCTGGAGG AGGAGAGCTG TGCGGACGCC

          1751                                                      1800
clone11   CAGGACGGGG AGCTGGACGG GCTCTGGACG ACTATCTCCA TCTTCATCAC
clone1    CAGGACGGGG AGCTGGACGG GCTCTGGACG ACTATCTCCA TCTTCATCAC

          1801                                                      1850
clone11   GCCCTTCCTG CTCAGCGTCT GCTACAGCGC CACCGTGACC CTCTTCAAGG
clone1    GCTCTTCCTG CTCAGCGTCT GCTACAGTGC CACCGTGACC CTCTTCAAGG
```

Figure 2(c)

```
          1851                                                      1900
clone11   TGGGGGTCCA CCCTGCTGGG CCCTCGGGCC CCCTCTCTGT CCCCAGGGTC
clone1    TGGGGGCCCA CCCTGCTGGG CCCTCGGGCC CCCTCTCTGT CCCCAGGGTC

          1901                                                      1950
clone11   CCCGCAGAGT CCCTCCCTGC CCCTCACTGT CCCTCCCTGT CCCTCTCTGT
clone1    CCCGCAGAGT CCCTCCCTGC CCCTCACTGT CCCTCCCTGT CCCTCTCTGT

          1951                                                      2000
clone11   CCCTCTCTGT CCCTCTCTGT CCCTCTCTGT CCGTTCATTT TCCCTTCACC
clone1    CCCTCTCTGT CCCTCTCTGT CCCTCTCTGT CCGCTCATTT TCCCTTCACC

          2001                                                      2050
clone11   GTAAGCTTGA GACAGATTGG GGTCATTTCA GAGGGCGTCT GAAGAGTCTC
clone1    GTAAGCTTGA GACAGATTGG GGTCATTTCA GAGGGCGTCT GAAGAGTCTC

          2051                                                      2100
clone11   TGTGCCGCAC GCCTCCCTTC ATGTCAGTGG GGAGAATTCA GCAAGGGTGG
clone1    TGTGCCGCAC GCCTCCCTTC ATGTCAGTGG GGAGAATTCA GCAAGGGTGG

          2101                                                      2150
clone11   AGTGCTGGGT GAGAAATGAG GCTTGCGGCG CTCACGAGCA GTGATGGGGC
clone1    AGTGCTGGGT GAGAAATGAG GCTTGCGGCG CTCACGAGCA GTGATGGGGC

          2151                                                      2200
clone11   ACTGCTGCTC CCTGAGACCT GCGCGGACAC CGTTTTCCAT CGCAGGAGAA
clone1    ACTGCTGCTC CCTGAGACCT GCGCGGACAC CGTTTTCCAT CGCAGGAGAA

          2201                                                      2250
clone11   GCGGGCAAGG GAAAACGCCC TCTTGGTCTC TCTTGAGTAA ATGTCGCGTT
clone1    GCGGGCAAGG GAAAACGCCC TCTTGGTCTC TCTTGAGTAA ATGTCGCGTT

          2251                                                      2300
clone11   TTGGTCATCA GTCCCTCCCC CAGTGAGGCT AGAGGAGTTT ACTTCTCCCT
clone1    TTGGTCATCA GTCCCTCCCC CAGTGAGGCT AGAGGAGTTT ACTTCTCCCT

          2301                                                      2350
clone11   CTCGATGGTC AGGTCAGGAC TGTCATAGAC TCCGGATCAC CTTCCTGTAA
clone1    CTCGATGGTC AGGTCAGGAC TGTCATAGAC TCCGGATCAC CTTCCTGTAA

          2351                                                      2400
clone11   ATGCTTGCTT TTTGTGTGCA GAGAGCCTGT TTTAGCTCGG GGGTCCTCAG
clone1    ATGCTTGCTT TTTGTGTGC. .AGAGCCTGT TTTAGCTCGG GGGTCCTCAG

          2401                                                      2450
clone11   CTCACTGAGC TCGCGGGGCA GGGGTGGGCT CGGGCTGGCG CCGCCTGTTC
clone1    CTCACTGAGC TCGCGGGGCA GGGGTGGGCT CGGGCTGGCG CCGCCTGTTC
```

Figure 2(d)

```
          2451                                                      2500
clone11   GGGAGCGCAT CTCCAGCATG CTGTCGCACA GCTTCGTTGC TAACAAGACC
clone1    GGGAGCGGCA TCTCCAGCTG CTGTCGCACA GCTTCGTTGC TAACAAGACC

          2501                                                      2550
clone11   GCTTAGTCTC GTGGTTAGAC CAACCTGCTT TCTCGAGTAA TTGTTAATTT
clone1    GCTTAGTCTC GTGGTTAGAC CAACCTGCTT TCTCGAGTAA TTGTTAATTT

          2551                                                      2600
clone11   ACAGGAGTTT CCTGTATTTT TCAACTTATA ATCCCCTAGT CAGATAACTC
clone1    ACAGGAGTTT CCTGTATTTT TCAACTTATA ATCCCCTAGT CAGATAACTC

          2601                                                      2650
clone11   TTTAATCACC TATTCTGCCC CTTCATTTTC TCCCTATCGA TCTCAGCAAC
clone1    TTTAATCACC TATTCTGCCC CTTCATTTTC TCCCTATCGA TCTCAGCAAC

          2651                                                      2700
clone11   CCATCACTGC CCTCACTGTC CTTAAACTGT CCCTTAACTG ACCAGACTGT
clone1    CCATCACTGC CCTCACTGTC CTTAAACTGT CCCTTAACTG ACCAGACTGT

          2701                                                      2750
clone11   CCCTCAGTGT CCCCTCAGAG TCACCTCCCT ATCACCTCAC TGTCCCTCTC
clone1    CCCTCAGTGT CCCCTCAGAG TCACCTCCCT ATCACCTCAC TGTCCCTCTC

          2751                                                      2800
clone11   TGCCCCTCTC TGCCCCTCTC TGTCCCTCCC TGCCCCTCCC CGTCCCCTCT
clone1    TGCCCCTCTC TGCCCCTCTC TGTCCCTCTC TGTCCCTCCC CGTCCCCTCT

          2801                                                      2850
clone11   CTGTCCCTCT CTGCCCCTCA CTGCTCCTCT CTGCACCTCA CTGCTCCTCA
clone1    CTGTCCCTCT CTGCCCCTCA CTGCTCCTCT CTGCACCTCA CTGCTCCTCA

          2851                                                      2900
clone11   CTGCCCTGGG GGAGGCCCGC ATCGAGGTGT CTCTGCTCAC CCCGTCCCCC
clone1    CTGCCCTGGG GGAGGCCCGC ATCGAGGTGT CTCTGCTCAC CCCGTCCCCC

          2901                                                      2950
clone11   ACCCCGTACC CCCCGCCAGG [redacted]
clone1    ACCCCGTCCC CCCCGCCAGG [redacted]
```

Figure 2(e)

Figure 3(a)-(b). Novel 3'prime flanking sequence (SEQ ID NO: 10) of rabbit Cgamma gene.

TGTGCACCCCGCACACAAATAAAGCACCCAGCTCTGCCCTGAGAGGCTGTCCTGATTCCT
TCCAAGGCAGAGGCTTCCACTCGGGCCGGACAGGGTTGGGGGGGGCCGTGGGCTCTGCT
GTGGCCAGCAGCCAGAACGGTCAACAGTGGGACAGGGGCAGACCCACAGCACGGGGCCT
GCCAAGAACTGGGCTCAGCCGGAGTGCTGTGGCAGGTCCCCCCTTGCAGCTAGCACGTGT
GTGCTGGGCAGGCAGAGGCCCCCAGGGGAGGAGCACACAGCTACCACCTCTGGAAGAGCC
TGGCCTGGCGCCCAGGTCCCAGTCCACAGGGTGTGTAGTACACAGAGCCTCATCTTACCA
CAGATGTAGGGACAGACCCACCACGCCCCTGCACCCCACCCAGCCTCGCCCCTTGTGGGA
CCAGGGCTACCACTCCACTCCCCCGCCCAGAGCAGCAGAAGCAGGTGGCATCCTCAGCAG
AGGGACAGTCTCACCCCTCCACGGCACTGAGCCCTGACCCATCAAACAAGCCCCTCCTGC
TGCACAGCACCTGTGTGCACATCACACACACACACACACACTGAGGCCTGACCCCA
TCAAACAAGCCCCTCCTGCTGCACAGCACCTGTGTGCACATCACACACACACACACACAC
ACACTGAGGCCTGACCCCATCCTGCCCTCCTGCTGCATGGCACCTGTGTGCACATCACAC
ACACATGCACACACACACTCACACACACTGAGCCCTGACCCCATCCTGCCCTCCTGCTGC
ATGGCACCTGTGTGCACATCACACACACACACACACACACACACACACTGAGCCCTGACC
CCATCCTGCCCTCCTGCTGCATGGCACCTGTGCACACATCACAAAACACGCCTGCCTCATA
CACTGGCACTCAGAAGGGGCCCCTGTACAGCATACACATGCACACACCTTGACACATGG
GCCCCCTACACACGCATCACACACACTCATGCACACTCCTCACACATGGCCCTCCTGCAC
ATACATTGCACACACACATGTGCACAGACCTCACAATGGGCCCCTGCACACACATTGTACAC
ACGCATTGTGCACACTCTTCACACATGGGCCCCCTGCACATGCATTGCACACACAGACACA
CACATGTGCATTCCTCACACATTGGGGCCTTGCAAGGATGCCCTGCACACACATTGCAC
ATGCTCACATGTGCACACACCCCACACTGGAGCCTTGCATAGGGCCCCCTGTACACACAC
CATGCATACACACACACCTCACACAAGGGGCCCCTACATACGCAAAACACACACACACA
CATGCACACACCTCATACACGGGCCCCCTACACACATCACACACACACACACACACGT
ATGCATGCCTCACACACAGACCTTGCAAGGGGCCCCCTGCACATGCATTCAACACATATG
CACATGTTTCACACACACGGTCCCCTACACACACTGCACACGCACACATGTGTACATGCT
TCACACACTGGGGCCTTGCATGGGGTCCCCTGCATAGCATAGCACCCAGAGCCACGCCAG
GTGCCTGGGCACATGGACACTGGTGCACACACAGCACCCAAGCCCAGCTCTCCCATCCAA
GGGGCACCAGCACCCCCCACTCACGAGCACCCTGAATTCCTGCTCCCCACAAGCGAAGGT
GCACCCTACCTCTCCAGACGTCCCTTTCCTGTGGCCACTCCCATAGGTATTGGCGAGACC
CTCCCTTGACCCTTGGGCCTGGTCACCCAGGGGACAGGAGAGGGCCAAGTTGGGCCACAG
TACCACTGCCCAGCAGGGGTGAGGCAAGCAGAGGGTGGGTCTGTGAGGCGTCTGGCCAGC
CGTGCTGGGGCCCAGGTGGGGAGCAGCTGGGTGGCTGAGGTGGCTTCCTTGCAGGTGGTT
GGGGGGAGCTGGCCCCACAAGTGCCACTGCCCAGCACTGTCCAGTGCTTCCCCTGAACC
TCCCGGCCACCCATCCCCAGCTGCAGCCGCAGAGGGAGTGCCCCTCGGCCTCCTCGGCAA
GACGCACGCTGACTGCCCCTCCCCATCCAGAGCTGCAGCTGGACGAGAGCTGTGCCGAGG
CCCAGGACGGGGAGCTGGACGGGCTGTGGACCACCATCACCATCTTCATCTCCCTCTTCC
TGCTCAGCGTGTGCTACAGGCCACAGTCACCCTCTTCAAGGTGGGTGCTGCACCCGGCA
CGGGTGGGCTGGGGGCCAGGGGCGGGGGCCGGGGGCCAGGCCCTCCTCACCCGCGCCGC
CGGTGCTGCAGGTGAAGTGGATCTTCTCGTCCGTGGTGGAGCTGAAACACACCATCGCTC
CCGACTACGAGGAACATGATCGGGCAGGGGGCCTAGGCCCTTCGTTCTCACAGCCTGCCTC
CCTGGCCAGCAGGAGCCCCCGCCTCCGCCTCGGACCCCATGGCTCTCTGCTCTGGCCGCT
CCGGACCCCTCCGCCTCGGGAGAAGCGCGCAGCTGATGCCTGCCGGCCCCTCCACGCAGC
AGTGCGGACAGCACGCATCTTGTCGTCCACCCGGCAGGACCCCACCCAGGGCCAGCCCTGA
CCGCCAGCCTCCTGGACTCAGGGCTCCTCTGAGAAAGGCCCACTTGTTGGTCCCCTCAG
CCCACACCCAGGCAGCCTCCGGTGGGTGCTTCCCTGGACCCCAGCCTGAGGCCTATGCTT
GTTCTCCTGTGGCTCTTACTCAGAGGCCCGTGCTGGACTCCCACCCACAGGGACAGTGCC

Figure 3(a)

CTGCTCCAACCCTCACTGCACTGGGGGTCATGGGGCCACCTTCTGTGCAGGGGTCCTGGC
TCCAAGGAGAACACTCGAAGGGCCTGCTTGGCCACCTGGCACCACGGGAGCCCCGCTGGG
TAGCTTGGCAGGGACCCCTGAGTAGAGGTGGGTGCACCCAGCCAGAAAGCCTGCTGGATG
GACAGGAGCCTGGCGTCCGGGCCCCAGGCAGGCAGACACGGCTTCATGGACAGGAGAGGC
CAAGGAACATCAGCAAAGAGAGACAGCTGGGCCGGGCGTTCCAGCCAGACCCATCCTGCA
GCCCAGCATCGGCC[redacted]

Figure 3(b)

Figure 4. Novel nucleotide sequence (SEQ ID NO:11) 3'prime of the rabbit Ckappa 1 gene.

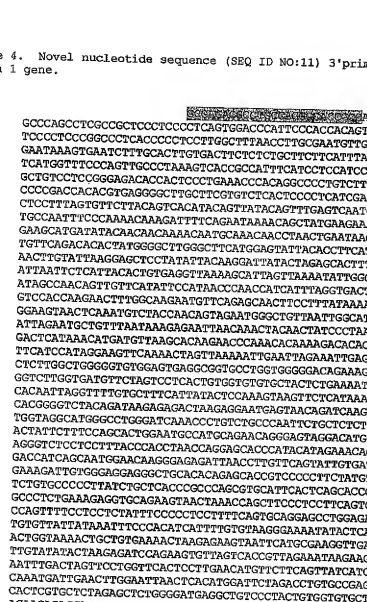

Figure 5. Novel nucleotide sequences (SEQ ID NO:12 and SEQ ID NO: 13) 5'prime of the rabbit Cgamma gene. The sequences between SEQ ID NO: 12 and SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

[redacted]CCGCTGAGTCTCACGCACCCCCG
GTGCAGGCAGTCCGGCCTCACCTGGAAGGTGCACTGACTGAAGACACTGCAAGGGGTGAG
AGCATTTCTCAGGAAAGAGCCCTGAGTTTAGAAGGCCAGAGAGCAGAGGGCTGAGGGCTG
CCTTGCGCTGCAACCCATGGAAACACAGGCTTAGCAGATGTTCAAGCTCCGGGAGTCCAC
ACTGGGTGAGGGCAGGCGTCCAGCCTGACATGGCCCCACAGACTCGCCCACAGGTGACG
CCAGATGAGGACGGTCAAGGATCGGGGGATCCTACATGCCCAGGGGCACCAAGACAGCCA
GGAGAGCACCAGAGGCCACAAGAGAGGCCTGGGACAGTCTCCCTGCTGACATCCAGAGCC
CAGGCCCCACTTGGCAGAGCTGGCTGAGAACACGTCTCTGCGGTGGAAGCTGCCCGTCC
TGGGTGTTGCTCGGCGGGCTAAGCCGACTGACGCGGGCGGGCCAGGCCATCGGCCCCAC
GGCCTGCAGCTTCCTCCCCAGCCCAGGCCACGTGGGCTCCTGGCTGAACTGGCCGCTCGC
TGAGCTCTCACCCCCCCACCCAGCAGCAGGCCGGGCGGTGCTGCCATGAGCTCCATTCCC
ACCACACAAGCGACAGCCCGGGCAGCGCCCCAGGCCCACGGGGCGTTTGCTGTGCGGCTC
GCACTCGCTGCTCAGGGCCAGCGCAGGGTGCAGCAGGGACTCACCAACCCGCCCCGACTC
GGCTGGCACGTTACTGGAGGCCTCTGAGCCTGACCGTGGCAGTGGGGCCCGAGCAGGCT
CCAGGCTGCCCCCTGCACCCTGGGCTTGCCGCTCCGGGACCCCTGGTGGGCACCTTCCCA
GATGTGCTCCCACCGTGCCTCCTTGGGGCTCTGGGCTCATAGCGGTCACTCTCCGCCTTC
TCTCCTCCCAGCCCTTTCCTGCCTCCCTATGGCCCCATCTAGCTCTGCCCTNTCTAGAGC
CTCTACCTGGAAGGAATCTGCTGTTGGACCAAGACACCACCCGCAGCACAGGTGGGCGCC
TTGCACTGTGCTAGGCCCTCCCCGCACAGAAAAGGGCCCTAGGCTCTGGAGGCTGCTGCT
GNCTCTGGGGCTGGCATCGGGCGCACCCTGCACCCTGCACCCTGAGGAAACTCAGGCCTG
CCCGCTCCAGGCCTGTCCCT

Gap of about 1000 nt

GAAGCTTTACTTGTTGGGGGCGG
GCAGGTCTAAGGGACCTGCCAGGTGTGGGGGCTGGGCTTGACTCAGCAGGAGCCTTCTAG
AAGGAAAGCTCTGGAGAAGGTGGGGGCAGAGGGCGGGAAAGGCCTGTGAGGAGGCGGGTG
GTGGGCAGGGCCACTGGGAAGGGAGGGCTGGGGGTGACACTCAGGTTGGCACTGGGGAGG
ACCTGAGGAGGCAGGTGCCAGGCACAGACGCTGAACCTGGGCAGGGCAGGGGCAGGTAACA
AGAAGGATTCTCCTTGGAGCCTGGTCCAGGGTGGTCCAGGCGGTCCAGGGCCTGGGGTT
TGCAAGCTGGGCTGTGACAGGGCCTCTCTCCCCAGGGGCAAGCAGCAAAGCCTGGGCACA
GAGCCCAAAGCCCCCACACAGAGAAGCTCCCCAGGGCAGGCCTGCAGGGCTTGGGGGAC
CTTCTTGGAGCAGGCAGAGGACAGAGGCATGAGATCAGCCTCCCAGAGGCTGGAATGATA
GGTCCAGCAGGAGGGGCCCACATGGGCTCTGGTTAGCAGGAGAAAACAGCCCCCAGGTCC
CCATGGCCACCACGCACCGACTGCTGGTGAAGCTTTGGGTGGCAGACGAGAGCCACATGG
CAGCTGCTCCTGTCACT[redacted]

Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the the M1 and M2 regions 3' of the Cgamma gene.

M1

```
                1                                                  46 SEQ
         camel  EPLLEEESCA EAQSGELDGL WTTISIFITL FLLSVCYSAT VTLFK. 14
     human-Ig3  .ELQLEESCA EAQDGELDGL WTTITIFITL FLLSVCYSAT VTFFK. 15
   human-Ig3/2  .ELQLEESCA EAQDGELDGL WTTITILITL FLLSVCYSAT VTFFK. 16
     human-Ig1  .ELQLEESCA EAQDGELDGL WTTITIFITL FLLSVCYSAT VTFFK. 17
     mouse-Ig1  .GLQLDETCA EAQDGELDGL WTTITIFISL FLLSVCYSAA VTLFK. 18
    mouse-Ig2a  .GLDLDDVCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK. 19
    mouse-mRNA  PGLQLDETCA EAQDGELDGL WTTITIFISL FLLSVCYSAA VTLFK. 20
     mouse-Ig3  .ELELNETCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK. 21
   mouse-Ig3/2  .ELELNGTCA EAQDGELDGL WTTITIFISL FLLSVCYSAS VTLFK. 22
 sheep-clone11  .LLLEEESCA DAQDGELDGL WTTISIFITP FLLSVCYSAT VTLFK. 23
  sheep-clone1  .LLLEEESCA DAQDGELDGL WTTISIFITL FLLSVCYSAT VTLFK. 24
  cow-clone11   .LLLEEEICA DDLDGELDGL ..........  ..........  ......  25
  cow-clone3/5  .LLLEEEICA DAQDGELDGL ..........  ..........  ......  26
        rabbit  ..LQLDESCA EAQDGELDGL WTTITIFISL FLLSVCYSAT VTLFK. 27
```

M2

```
                1                   27                             SEQ
         camel  VKWIFSSVVE LKRTIVPDYR NMIGQGS                       28
     human-Ig3  VKWIFSSVVD LKQTIIPDYR NMIGQGA                       29
   human-Ig3/2  VKWIFSSVVD LKQTIIPDYR NMIGQGA                       30
     human-Ig1  VKWIFSSVVD LKQTIIPDYR NMIGQGA                       31
     mouse-Ig1  VKWIFSSVVE LKQTLVPEYK NMIGQAP                       32
    mouse-Ig2a  VKWIFSSVVE LKQTISPDYR NMIGQGA                       33
    mouse-mRNA  VKWIFSSVVE LKQTLVPEYK NMIGQAP                       34
     mouse-Ig3  VKWIFSSVVQ VKQTAIPDYR NMIGQGA                       35
   mouse-Ig3/2  VKWIFSSVVQ VKQTAIPDYR NMIGQGA                       36
        rabbit  VKWIFSSVVE LKHTIAPDYR NMMGQGA                       37
sheep-clone1/11 VKWIFSSV.. ..........                              38
```

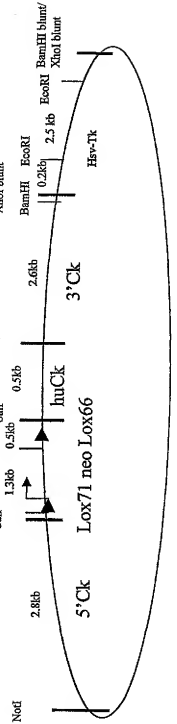

Figure 7a: DNA construct for the replacement of rabbit Ck with human Ck. A 0.5 kb fragment containing a DNA sequence encoding human Ck is flanked by sequences from the rabbit Ck1 gene. The upstream sequence (5'Ck) is 2.8 kb, the downstream sequence (3'Ck) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk cassette for negative selection.

Figure 8. DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cγ1 gene segment flanked by 50 nucleotides derived from the rabbit heavy chain immunoglobulin gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

tgacctacct accctgccaa ggtcaggggt cctccaaggc
aagggatcac atggcaccac ctctcttgca gcctccacca agggcccatc ggtcttcccc
ctggcaccct cctccaagag cacctctggg ggcacagcgg ccctgggctg cctggtcaag
gactacttcc ccgaaccggt gacggtgtcg tggaactcag gcgccctgac cagcggcgtg
cacaccttcc cggctgtcct acagtcctca ggactctact ccctcagcag cgtggtgacc
gtgccctcca gcagcttggg cacccagacc tacatctgca acgtgaatca caagcccagc
aacaccaagg tggacaagaa agttggtgag aggccagcac agggagggag ggtgtctgct
ggaagccagg ctcaggcgtc ctgcctggac gcatcccggc tatgcagccc cagtccaggg
cagcaaggca ggccccgtct gcctcttcac cggaggcct ctgcccgccc cactcatgct
cagggagagg gtcttctgga tttttccca ggctctgggc aggcacaggc taggtgcccc
taacccaggc cctgcacaca aaggggcagg tgctgggctc agacctgcca agagccatat
ccgggaggac cctgcccctg acctaaggcc accccaaagg ccaaactctc cactcctca
gctcggacac cttctctcct cccagatttcc agtaaatccc aatcttctct ctgcagagcc
caaatcttgt gacaaaactc acacatgccc accgtgccca ggtaagccag cccaggcctc
gccctccagc tcaaggcggg acaggtgccc tagagtagcc tgcatccagg gacaggccc
agccgggtgc tgacacgtcc acctccatct cttcctcagc acctgaactc ctgggggga
cgtcagtctt cctcttcccc ccaaaaccca aggacacct catgatctcc cggacccctg
aggtcacatg cgtggtggtg gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt
acgtggacgg cgtggaggtg cataatgcca agacaaagcc gcgggaggag cagtacaaca
gcacgtaccg tgtggtcagc gtcctcaccg tcctgcacca ggactggctg aatggcaagg
agtacaagtg caaggtctcc aacaaagccc tcccagcccc catcgagaaa accatctcca
aagccaaagg tgggacccgt ggggtgcgag ggccacatgg acagaggccg gctcggccca
ccctctgccc tgagagtgac cgctgtacca acctctgtcc ctacagggca gccccgagaa
ccacaggtgt acaccctgcc cccatcccgg gatgagctga ccaagaacca ggtcagcctg
acctgcctgg tcaaaggctt ctatcccagc gacatcgccg tggagtggga gagcaatggg
cagccgggaa acaactacaa gaccacgcct cccgtgctgg actccgacgg ctccttcttc
ctctacagca agctcaccgt ggacaagagc aggtggcagc aggggaacgt cttctcatgc
tccgtgatgc atgaggctct gcacaaccac tacacgcaga agagcctctc cctgtctccg
ggtaaatgag cgctgtgccg gcgagctgcc cctctccctc ccccccacgc cgcagctgt.

Figure 9. The DNA fragment (SEQ ID NO: 52) containing a VH gene segment with more than 80% sequence identity with rabbit VH elements and encoding a human VH element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

tgagtgacag tgtcctgacc atgtcgtctg tgtttgcagg tgtccagtgt
gaggtgcagc tgttggagtc cgggggaggt ctcgtccagc caggggggac cctgagactc
acctgcgcag tctctggatt caccttcagt agctatgcaa tgagctgggt ccgccaggct
ccagggaagg ggctggaatg ggtcggagcc attagtggta gtggtagcac atactacgcg
gacagcgtga aaggccgatt caccatctcc agagacaact ccaagaacac gctgtatctg
caaatgaaca gtctgagagc cgaggacacg gccgcctatt actgtgcgaa agacacagtg
aggggccctc aggctgagcc cagacacaaa cctccctgca

Figure 10. DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin light chain Cκ gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappal gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

.catccacat ggcacccagg
ggagatgtcc actggtacct aagcctcgcc atcctgtttg cttctttcct caggaactgt
ggctgcacca tctgtcttca tcttcccgcc atctgatgag cagttgaaat ctggaactgc
ctctgttgtg tgcctgctga ataacttcta tcccagagag gccaaagtac agtggaaggt
ggataacgcc ctccaatcgg gtaactccca ggagagtgtc acagagcagg acagcaagga
cagcacctac agcctcagca gcaccctgac gctgagcaaa gcagactacg agaaacacaa
agtctacgcc tgcgaagtca cccatcaggg cctgagctcg cccgtcacaa agagcttcaa
caggggagag tgttagagcg agacgcctgc cagggcaccg ccagcgaccc tgaggcccag
cctcgc.

Figure 11. DNA fragment (SEQ ID NO: 54) containing a Vκ gene segment with more than 80% sequence identity with rabbit Vκ elements and encoding a human Vκ element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

..ggcaggct gctcccaccc
catgcaggag gcagtaccag gcaggaccca gcatggacat gagggtccct gctcagctcc
tggggctcct gctgctctgg ctcccaggta aggagggaaa caacaaaaat tttattcagc
cagtgtagcc actaatgcct ggcacttcag gaaattcttc ttagaacatt actaatcatg
tggatatgtg tttttatgtt cctaatatca gataccagat gttacatcca gatgacccag
tctccatcct ctctgtctgc atctgtggga gacagagtca ccatcacttg ccgagccagt
cagggcatta gcaattactt agcctggtat cagcagaaac cagggaaggt tcccaagctc
ctgatttatg ctgcatccac tttgcaatct ggggtcccat cgcggttcag tggcagtgga
tctgggacag atttcactct taccatcagc agcctgcagc ctgaagatgt tgccacctat
tactgtcaaa agtacaacag tgcccctcca ctttttggcg gagggaccaa ggtggagatc
aaacgtaagt gcactttcct aatgttcctc accgtttctg cctgatttgt ttgcttttc
catttttcgctat..

Figure 12. DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides derived from the chicken light chain gene. The DNA sequence of chicken origin is underlined.

catacacag ccatacatac gcgtgtggcc gctctgcctc tctcttgcag gtcagcccaa
ggctgccccc tccgtcactc tgttcccgcc ctcctctgag gagcttcaag ccaacaaggc
cacactggtg tgtctcataa gtgatctcta cccgggagcc gtgacagtgg cttggaaagc
agatagcagc cccgtcaagg cgggagtgga gaccaccaca ccctccaaac aaagcaacaa
caagtacgcg gccagcagct atctgagcct gacgcctgag cagtggaagt cccacagaag
ctacagctgc caggtcacgc atgaagggag caccgtggag aagacagtgg ccctacaga
atgttcatag tagtcccact ggggatgcaa tgtgaggaca gtggttcctc accctccctg

Figure 13. Modification of the chicken light chain locus using the ET system.
A chicken genomic BAC clone with the full length light chain locus was modified by homologous recombination. In a first step Cλ was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human Cλ gene. The homology stretch was 50bp.

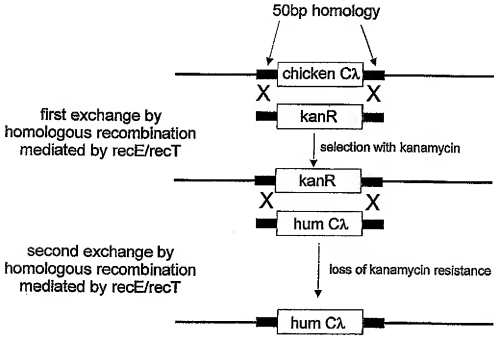

Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived form chicken immunolgobulin DNA sequences are underlined.

.ttgccgttt tctcccctct ctcctctccc tctccaggtt ccctggtgca gtcagtgctg actcagccgc
cctcggtgtc agcagccccg ggacaagaag tcacgatctc ctgctccggg tctagtagca acattggcga
taattttgtc tcttggtacC agcagctgcc tggcactgcc cctaagcttc tgatctatga taacaacAag
agaccctcgg gcatccctga ccgattctcc ggttccaaat ccggcacctc agccacatta ggcatcactg
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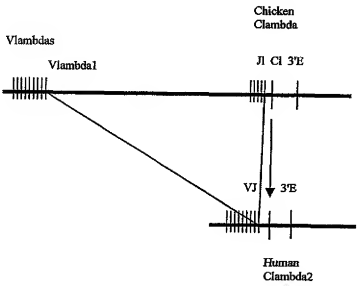

Figure 15. Humanized chicken light chain locus.